

**THE EFFECTS OF CHRONIC MELATONIN TREATMENT ON
MYOCARDIAL FUNCTION AND ISCHAEMIA AND REPERFUSION
INJURY IN A RAT MODEL OF DIET-INDUCED OBESITY**

Frederic NDUHIRABANDI

Faculty of Health Sciences
Department of Biomedical Sciences
Division of Medical Physiology
Stellenbosch University

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Promotors: Prof E F du Toit
Prof A Lochner

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ABSTRACT

Introduction:

Obesity is a major risk factor for ischaemic heart disease. Obesity-induced metabolic abnormalities have been associated with increased oxidative stress which may play an important role in the increased susceptibility to myocardial dysfunction and ischaemia-reperfusion (I/R) injury seen in obesity. The pineal gland hormone, melatonin, has powerful antioxidant properties. Previous studies have shown that short-term or acute melatonin administration protects the normal healthy heart of lean animals against I/R damage. However, the effects of melatonin on the heart in obesity remain unknown. Moreover, the myocardial signalling mechanisms associated with the cardioprotective effects of melatonin have not been established.

Aim:

Using a rat model of diet induced obesity, we set out to: 1) investigate the effects of chronic melatonin administration on the development of diet-induced systemic alterations including biometric and metabolic parameters and oxidative stress, 2) determine whether chronic melatonin treatment protects the myocardium against ischaemia-reperfusion injury, and 3) determine whether melatonin treatment confers cardioprotection by altering the reperfusion injury salvage kinase (RISK) pathway signalling and the pro-apoptotic p38 MAPK, AMPK and GLUT-4 expression.

Methods:

Male rats weighing 200±20g were randomly allocated to four groups: 1) C, control rats receiving a standard commercial rat chow and drinking water without melatonin; 2) CM, control rats receiving melatonin (4mg/kg/day) in drinking water; 3) D, diet-induced obesity rats, receiving a high calorie diet and drinking water without melatonin; 4) DM, diet-induced obesity rats, receiving melatonin in drinking water. After 16 weeks of treatment and feeding, rats were weighed and blood and myocardial tissue collected to document biochemical and molecular biological changes. Hearts were perfused on the isolated working rat heart perfusion apparatus for the evaluation of myocardial function and infarct size. The Reperfusion Injury Salvage Kinases (RISK) pathway (PKB/Akt (Ser-473), ERK p42/ p44) and p38 MAPK (mitogen-activated protein kinase) were investigated in pre-and post-ischaemic hearts using Western blotting techniques. Post-ischaemic activation of AMPK (5'AMP-activated protein kinase) (Thr-172) and GLUT-4 (glucose transporter) expression were also investigated. Serum and baseline myocardial glutathione (GSH) content were measured. In addition, serum lipid

peroxidation products: thiobarbituric reactive substances (TBARS), conjugated dienes (CD) and lipid hydroperoxide (LOOH), were also determined.

Results:

The high-calorie diet caused increases in body weight, visceral adiposity, heart weight, serum insulin, leptin, blood triglycerides, and low HDL-cholesterol levels. Blood glucose levels were similar for both diet fed rats and controls. Myocardial glutathione, serum glutathione, total cholesterol, TBARS, LOOH, CD as well as total cholesterol (TC) levels were not affected by the high calorie diet. Chronic melatonin treatment reduced body weight gain, visceral adiposity, heart weight, blood triglycerides, serum insulin, HOMA index, serum leptin (DM vs D, $p < 0.01$), and increased blood HDL-C in diet treated rats while there was no effect on these parameters in control rats, despite the reduction in body weight, heart weight and visceral adiposity. Melatonin treatment had no effect on myocardial or serum GSH and LOOH in either control or diet animals. It however reduced TBARS and CD in the diet and control groups, respectively. At baseline, chronic melatonin treatment caused a significant increase in phospho-PKB/total PKB ratio and a concomitant reduction in phospho-p38 MAPK/total p38 MAPK ratio of control hearts while there were no such effects on diet-induced-obesity hearts. Infarct size was significantly reduced by melatonin in both diet and control groups (DM: $16.6 \pm 2.0\%$; D: $38.4 \pm 2.6\%$ ($p < 0.001$), and CM: $12.8 \pm 1.5\%$; C: $30.4 \pm 1.0\%$, $p < 0.001$). After coronary artery occlusion and 30 minutes of reperfusion, melatonin increased percentage recovery of aortic output (DM: $28.5 \pm 6.5\%$; D: $6.2 \pm 6.2\%$, $p < 0.01$), cardiac output (DM: $44.4 \pm 5.2\%$; D: $26.6 \pm 5.1\%$, $p < 0.01$) and total work (DM: $34.5 \pm 5.6\%$; D: $20.4 \pm 7.9\%$, $p < 0.05$) of diet-induced obesity hearts, while having no effect on control hearts. During reperfusion, hearts from melatonin treated rats had increased activation of PKB/Akt ($p < 0.01$), ERK42/44 ($p < 0.05$), and reduced p38 MAPK activation ($p < 0.05$). There was no difference in post-ischaemic activation of AMPK (Thr-172) and GLUT-4 expression in either control or diet fed rats.

Conclusions:

We successfully demonstrated that chronic melatonin treatment prevented the development of diet-induced metabolic abnormalities and improved *ex vivo* myocardial function. Melatonin protected the heart against ischaemia-reperfusion injury that was exacerbated in obesity. This was achieved by activation of the RISK pathway. The antioxidant properties of melatonin were involved in these cardioprotective effects.

Key words: Antioxidant, cardioprotection, diet-induced obesity, melatonin, myocardial ischaemia-reperfusion injury, insulin resistance.

ABSTRAK

Inleiding

Vetsug of obesiteit is een van die hoof risikofaktore vir iskemiese hartsiekte. Obesiteit-geïnduseerde metaboliese abnormaliteite gaan met verhoogde oksidatiewe stres gepaard wat op sy beurt 'n belangrike rol mag speel in die miokardiale wanfunksie en verhoogde vatbaarheid vir iskemie-herperfusie (I/H) beskadiging, kenmerkend van vetsug. Melatonien, die hormoon afgeskei deur die pineaalklier, is 'n kragtige anti-oksidadant. Vorige studies het getoon dat kort-termyn of akute toediening van melatonien die normale hart van gesonde diere teen I/H beskadiging deur middel van sy anti-oksidadant aksies beskerm. Die effek van melatonien op die hart in obesiteit is egter nog onbekend. Hierbenewens is die miokardiale seintransduksie meganismes geassosieer met die beskermende effekte van die hormoon nog nie ontrafel nie.

Doelstellings

'n Model van dieet-geïnduseerde obesiteit in rotte is gebruik om die volgende te bepaal: (i) die effek van kroniese melatonientoediening op die ontwikkeling van dieet-geïnduseerde sistemiese veranderinge soos biometriese en metaboliese parameters en oksidatiewe stres (ii) die effek van kroniese melatonienbehandeling op die respons van die hart op I/H beskadiging en (iii) die rol van herperfusie beskadiging op die aktivering van PKB/Akt en ERK42/44 (die sg RISK seintransduksiepad), die pro-apoptotiese p38MAPK, AMPK sowel as die uitdrukking van GLUT-4.

Metodes

Manlike Wistar rotte (200±20g) is ewekansig in vier groepe verdeel: (i) C, kontrole rotte wat 'n standaard rotdieet en drinkwater sonder melatonien ontvang (ii) CM, kontrole rotte wat melatonien (4mg/kg/dag) ontvang (iii) D, dieet-geïnduseerde vet rotte wat 'n hoë kalorie dieet en drinkwater sonder melatonien ontvang (iv) DM, dieet-geïnduseerde vet rotte wat melatonien (4mg/kg/dag) in die drinkwater ontvang. Na 16 weke van behandeling, is die rotte geweeg, bloed en hartweefsel gekollekteer vir biochemiese en molekulêre biologie bepalings. Harte is geperfuseer volgens die werkhartmodel, blootgestel aan iskemie/herperfusie vir evaluering van funksionele herstel en infarkt grootte. Uitdrukking en aktivering van PKB/Akt (Ser-473), ERKp42/p44 en p38MAPK van pre-en postiskemiese hartweefsel is met behulp van Western blot bepaal. Postiskemiese aktivering van AMPK (5'AMP-aktiveerde proteïen kinase) (Thr-172) en GLUT-4 (glukose transporter) is op soortgelyke wyse bepaal. Serum en basislyn hartweefsel glutatioon (GSH) inhoud asook tiobarbituursuur reaktiewe substans (TBARS), gekonjugeerde diene (CD) en lipiedhidroperoksied (LOOH) konsentrasies is bepaal.

Resultate

Die hoë kalorie diet het 'n toename in liggaamsgewig, visserale vet, hartgewig, serum insulien, leptien, plasma trigliseried en lae HDL-cholesterol vlakke tewegebring. Bloed glukosevlakke was egter dieselfde in die vet en kontrole rotte. Miokardiale glutatioon, serum glutatioon, totale cholesterol, TBARS, LOOH, CD is nie deur die dieet beïnvloed nie. Chroniese melatonien behandeling het die liggaamsgewig, visserale vet, hartgewig, plasma trigliseried, serum insulien en leptien, HOMA indeks verlaag (DM vs D, $p < 0.05$) en die HDL-cholesterol verhoog in die dieetrotte, terwyl dit geen effek op hierdie parameters in kontrole rotte gehad het nie (uitgesonderd 'n afname in liggaamsgewig, hartgewig en visserale vet). Melatonien behandeling het geen effek op hart of serum GSH en LOOH in kontrole en vet rotte gehad nie. Dit het egter die TBARS en CD in beide vet en kontrole rotte verlaag. Chroniese melatonien toediening het 'n beduidende toename in basislyn fosfo PKB//totale PKB ratio en 'n afname in fosfo p38MAPK/totale p38MAPK ratio tewegebring in harte van kontrole rotte, maar soortgelyke effekte is nie in die harte van die vet rotte waargeneem nie. Infarkt grootte is beduidend deur melatonienbehandeling verlaag in beide dieet en kontrole groepe (DM: $16.6 \pm 5.2\%$, D: $38.4 \pm 2.6\%$ ($p < 0.001$); CM: $12.8 \pm 1.5\%$; C 30.4 ± 1.0 ($p < 0.001$). Na koronere arterie afbinding en 30 min van herperfusie, het melatonien die persentasie herstel van aorta omset (DM: $28.5 \pm 6.5\%$; D: $6.2 \pm 6.2\%$, $p < 0.01$), kardiaale omset (DM: $44.4 \pm 5.2\%$ D: $26.6 \pm 5.1\%$, $p < 0.01$) en totale werk (DM: $34.5 \pm 5.6\%$; D $20.4 \pm 7.9\%$, $p < 0.05$) in die harte van dieetrotte verbeter, terwyl dit sonder effek was in kontrole harte. Tydens herperfusie het harte van melatonienbehandelde rotte verhoogde aktivering van PKB/Akt ($p < 0.01$) en ERKp42/p44 ($p < 0.05$) getoon, terwyl aktivering van p38MAPK verlaag is ($p < 0.05$). Geen verskil in postiskemiese aktivering van AMPK en GLUT-4 uitdrukking is in beide kontrole en dieetrotte waargeneem nie.

Gevolgtrekkings

Ons het daarin geslaag om aan te toon dat chroniese melatonienbehandeling die ontwikkeling van dieet-geïnduseerde metaboliese abnormaliteite beduidend kan voorkom en ex vivo miokardiale funksie verbeter. Melatonien het ook die hart teen iskemie/herperfusie beskadiging beskerm in beide kontrole en dieetrotte. Bogenoemde veranderinge het met aktivering van PKB/Akt en ERKp42/p44 gepaard gegaan. Die anti-oksidant effekte van melatonien was heelwaarskynlik hierby betrokke.

Sleutelwoorde

Anti-oksidant; miokardiale beskerming; dieet-geïnduseerde vetsug; obesiteit; melatonien; iskemie/herperfusie beskadiging; insulienweerstandigheid.

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LIST OF ABBREVIATIONS

I. Units of measurement

%:	percentage	min:	minute
cm:	centimeter	mM:	millimolar
g:	gram	mm:	millimeter
Hg:	mercury	mmol:	millimol
IU:	International unit	°C:	degree celcius
kg:	kilogram	v:	volume
kj:	kilojoules	μ:	micro
L:	litre	μL:	microlitre
M:	molar	μm:	micrometer
mg:	milligram		

II. Chemical components

AA-NAT: Arylalkylamine N-acetyltransferase	COX-2: Cyclooxygenase- 2
ACC: Acetyl-CoA carboxylase	CREBP: cAMP-response element binding protein
AFMK: N-acetyl-N-formyl-5-methoxykynuramine	DAG: Diacylglycerol
AGE: advanced glycation end products	DCDHF: Dichlorodihydro-fluorescein diacetate
AMK: N1-acetyl-5-methoxy-kynurenine	DHPR : Dihydropyridine receptor
AMMC: 3-acetamidomethyl-6-methoxycinnolinone	ERK 42/44: Extracellular signal regulated kinase p42/p44
AMP: Adenosine monophosphate	FFA: Free fatty acids
Ang I or II: Angiotensin I or II	GLUT-4: Glucose transporter 4
ANP : Atrial natriuretic peptide	GPCR: G-protein coupled receptor
apoB: Apolipoprotein B	GPx: Glutathione peroxidase
ATP: Adenosine triphosphate	GSH: Reduced glutathione;
ATPase: Adenosine triphosphatase	GSK-3: Glycogen synthase kinase-3
Ca ²⁺ : Calcium	HDL: High density lipoprotein
cAMP: Cyclic adenosine monophosphate	IL-6: Interleukin-6
CAT: Catalase	IP3: Inositol 1,4,5-trisphosphate
cGMP: Cyclic guanosine monophosphate	IR: Insulin receptor
CO ₂ : Carbon dioxide	IRS: Insulin receptor substrate

LCFA: Long chain fatty acid
LDH: Lactate dehydrogenase;
LDL: Low density lipoprotein
MAPK: Mitogen activated protein kinase
MDA: Malondialdehyde
MPO: Myeloperoxidase;
MPTP: Mitochondrial permeability transition pore
MT1/MT2: Melatonin receptor 1 and 2
NADPH: Nicotinamide adenine dinucleotide phosphate
NO: Nitric oxide
ONOO⁻: Peroxynitrite
PAI-1: Plasminogen activator inhibitor-1
PDE2: Phosphodiesterase-2
PGE2: Prostaglandin-E2
PI3-K: Phosphatidylinositol 3 kinase
PKA: Protein kinase A
PKB/Akt: Protein kinase B
PKC: Protein kinase C
PLB: Phospholamban

PPAR: peroxisome proliferator activated receptor
RISK: Reperfusion injury salvage kinase
RNS: Reactive nitrogen species
ROOH/LOOH: Lipid hydroperoxide
ROS: reactive Oxygen species
RT-PCR: Reverse transcription-polymerase chain reaction
RyR: Ryanodine receptor
SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SERCA2a: Sarco-(endo)-plasmic reticulum Ca²⁺-ATPase2
SOCS3: Suppressor of cytokine signalling 3
SOD: Superoxide dismutase
STAT-3: Signal transducer and activator of transcription 3
TBARS: thiobarbituric acid reaction substance
TNF- α : Tumour necrosis factor alpha
TRIG: Triglyceride/Triacylglycerol
VLDL: Very low density lipoprotein

III. Others

AMI (MI): Acute myocardial infarction
AO: Aortic output
BW: Body weight
CAL: Coronary artery ligation
CO: Cardiac output
DIO: Diet induced obesity
DP: Diastolic blood pressure
e.g.: for example (*exempli gratia*)
ETC: Electron transport chain
HOMA: Homeostasis model assessment
HR: Heart rate
HW: Heart weight

i.e.: That is (*id est*)
i.p.: Intraperitoneal injection
I/R: Ischaemia and reperfusion injury
IFS: Infarct size
IPC: Ischaemic preconditioning
IPOC: Ischaemic postconditioning
LD: Langendorff
LV: Left Ventricle
LVDevP: Left ventricular developed pressure
LVEDp: Left ventricular end-diastolic pressure

MAP: Mean arterial pressure
MS: Metabolic syndrome
NCEPATP III: National Cholesterol Education
Program's Adult Panel III
OSAS: Obstructive sleep apnoea syndrome
PVDF: Polyvinylidene Fluoride
RAS /RAAS: Renin angiotensin system or
renin-angiotensin-aldosterone system
RIA: Radioimmunoassay

SCN: Suprachiasmatic nucleus
SEM: Standard error of the mean
SP: Systolic blood pressure
VF: Ventricular fibrillation
VT: Ventricular tachycardia
WH: Working heart
WHO: World Health Organization
Wtot: Work total or total work

CHAPTER ONE

INTRODUCTION

The prevalence of obesity continues to increase throughout the world. Obesity refers to an excess body fat accumulation that usually leads to health problems. People are defined as being obese if they have a BMI (body mass index) exceeding 30 kg/m². Overweight refers to a BMI between 25 and 30 kg/m². Obesity has been recognized as a public health problem of pandemic proportions affecting both developed and developing countries (Haslam & James, 2005). Kelly et al. (2008) analyzed the global burden of obesity in 2005 and found that at least 33.0% of the world's adult population (1.3 billion people) was overweight or obese. Among them 300 million were obese. It is predicted that up to 57.8% of the adult population (3.3 billion people) could be either overweight or obese by 2030, if the actual trend continues. Wang et al. (2009) reported that by 2030 more than 85% of United States (US) adult population would become overweight or obese with as many as 51.1% of them being obese. In the South African context, the prevalence of obesity has also reached epidemic proportions with almost 57% of the women and 29% of the men over the age of 30 years being identified as overweight or obese (Poane et al., 2002).

Together with this alarming prevalence, obesity is associated with increased risk for numerous co-morbidities including type 2 diabetes, hypertension, cardiovascular diseases, obstructive sleep apnoea syndrome, osteoarthritis and some cancers (Guh et al., 2009). These figures clearly indicate that the financial burden on the government and society will increase due to increased health care expenditures attributable to obesity and its related complications (Haslam & James, 2005). Importantly, obesity has been identified as an independent risk factor for cardiovascular diseases in general. Cardiovascular disease is the world's leading cause of morbidity and mortality and contributes to 29% of all deaths (16.7 million deaths) each year (Barry et al., 2008). Furthermore, metabolic syndrome which is the cluster of cardiovascular risk factors which include obesity, has also reached pandemic proportions. In most developed and developing countries between 20% and 30% of the adult population can be characterized as having the metabolic syndrome (Grundy, 2008)

Acute coronary occlusion results in ischaemic heart disease, the leading cause of morbidity and mortality in the Western world and some developing countries including South Africa.

Here, it is the fifth highest cause of mortality (WHO, 2009) while it is the leading cause of mortality in the Western Cape Province (Bradshaw et al., 2004). The clinical consequences of ischaemic heart disease include angina pectoris, acute myocardial infarction (AMI), chronic post myocardial infarction, heart failure and sudden cardiac death. Obesity and related metabolic abnormalities including insulin resistance, dyslipidaemia, and hypertension have been associated with myocardial infarction or necrosis of the myocardium (Fedorowski et al., 2009; Prasad et al., 2009; Ranjith et al., 2007). In addition, independently of other co-morbidities such as hypertension, atherosclerosis and myocardial infarction, an increased adiposity alone has been shown to impair both cardiac diastolic and systolic function (Mittendorfer et al., 2008). It has been demonstrated that obesity increases left ventricular hypertrophy and exacerbates myocardial susceptibility to ischaemia and reperfusion (I/R) injury (Du Toit et al., 2008). However, although obesity remains the driving force behind the prevalence of myocardial infarction, the link between obesity and myocardial infarction is complex and not yet fully understood.

The unifying hypothesis linking obesity (central obesity) and its cardiovascular complications is an increase in oxidative stress (condition characterized by an elevated reactive oxygen/nitrogen species production and insufficient antioxidant capacity) associated with a dysregulation of adipose tissue-derived proteins (adipocytokines), each predisposing the obese individual to the insulin resistance state (Furukawa et al., 2004; Vincent et al., 2006; Evans et al., 2002 & 2005). Excessive reactive oxygen/nitrogen species generation has been shown to play a crucial role in pathogenesis of cardiovascular disorders such as heart failure and myocardial ischaemia/reperfusion injury (Dhalla et al., 2000a&b; Molavi & Mehta, 2004; Ferrari et al., 2004). Therefore the use of antioxidants to treat obesity and its cardiovascular complications seems logical. In addition, the beneficial effects of antioxidant treatment on I/R-induced myocardial damage have been demonstrated (Sethi et al., 2000; Abe et al., 2008). Finally, oxidative stress has recently been shown to play a key role in the metabolic syndrome (Hopps et al., 2009; Roberts et al., 2009). Therefore, the prevention of an increase in oxidative stress may be crucial to stop the development of obesity-related metabolic complications.

Melatonin or N-acetyl-5-methoxytryptamine, the hormone which is primarily secreted by the pineal gland, has powerful antioxidant properties (Tan et al., 2007). Unlike other antioxidants, it is a small, highly lipophilic and hydrophilic molecule able to cross all morphological barriers and to act not only in every cell but also within every subcellular compartment (Pandi-Perumal

et al., 2006). Melatonin is involved in a wide range of physiological functions in humans and animals (Pandi-Perumal et al., 2006). Acute melatonin treatment protects the heart against I/R injury *via* both its direct free radical scavenging activities and its indirect actions in stimulating antioxidant enzymes (Reiter & Tan, 2003; Sahna et al., 2005; Petrosillo et al., 2009; Lochner et al., 2006; Genade et al., 2008). The long-term administration of melatonin also reduced myocardial infarction (Lochner et al., 2006) in lean animals, indicating its potential future therapeutic use. Chronic administration of melatonin was associated with amelioration of physiological changes associated with obesity (Hussein et al., 2007; Prunet-Marcassus et al., 2003; She et al., 2009), suggesting potential beneficial effects of melatonin in obesity management. However, to our knowledge, the effects of melatonin in obesity are incompletely elucidated. In addition, the effects of melatonin on the heart in obesity remain unknown. The preventive effects of chronic melatonin administration starting before the establishment of obesity have to be established.

In order to expand our knowledge of some of these outstanding issues, the effects of chronic melatonin treatment were investigated in rat model of diet-induced obesity. We focused on the effects of melatonin on myocardial function and ischaemia/reperfusion injury. Thus, to gain more insight into the overall effects of melatonin treatment in obese animals compared to lean animals, particularly on the ischaemic heart, we review the current literature on myocardial ischaemia/ reperfusion injury and the impact of obesity on cardiovascular disease including the obesity-induced systemic and cardiac alterations. The effects of melatonin on the heart as well as its actions in obesity are also reviewed. The present study adds knowledge gained in previous studies done on acute and short-term melatonin treatment on the heart. This is the first study to investigate the effects of chronic melatonin supplementation on the heart in obesity.

CHAPTER TWO

LITERATURE REVIEW

2.1. ISCHAEMIA-REPERFUSION INJURY: AN OVERVIEW

2.1.1. Concepts

The term “ischaemia” refers to an insufficient blood supply to any area of the body. When applied to the myocardium, it refers to the condition in which the blood flow to the heart muscle is diminished and thus the supply of oxygen and nutrients to the myocardium is inadequate or insufficient to maintain normal oxidative metabolism (Jennings & Yellon, 1992). The pivotal feature of this condition is that oxygen supply to the mitochondria is inadequate to support oxidative phosphorylation (Solaini & Harris, 2005). Clinical myocardial ischaemia generally results from the formation of atherosclerotic lesions in the coronary arteries and when this situation is not rectified, it leads to myocardial infarction (MI) or cell death (Opie, 2004).

Early reperfusion is necessary for maintaining cell viability and protecting the heart against myocardial infarction. However, reperfusion itself induces severe and irreversible damage to the myocardium and coronary arteries. This phenomenon is known as “myocardial reperfusion injury” (Yellon & Hausenloy, 2007). In most instances, the concept of ischaemia/reperfusion injury (I/R) refers to a mixture of injury induced by sustained ischaemia and reperfusion (Skyschally et al., 2008). This section briefly describes myocardial alterations/changes associated with ischaemia followed by reperfusion. The description of vascular alterations is beyond the focus of our study (for review see Carden & Granger, 2000 and Opie, 2004).

2.1.2. Ischaemic injury

Myocardial ischaemia results in damage of which the gravity (e.g., reversibility or irreversibility) depends on severity of the coronary flow reduction, and the length of the ischaemic insult as well as the location being affected (e.g., possibility of collateral flow). As Skyschally et al. (2008) have noted, it is technically impossible to determine in a distinct piece of myocardium whether the observed damage/injury (e.g., necrosis) is exclusively caused by ischaemia or reperfusion or the combination of both. The two forms of injury induced by ischaemia are defined as being reversible and irreversible damage.

2.1.2.1. Levels of injury

Depending on the duration of ischaemia, three levels of myocardial injury have been identified namely arrhythmias, contractile dysfunction and irreversible damage. With the first, short term ischaemia (less than 5 minutes) leads to an abrupt decrease in oxygen demand associated with a reduction in contractility as the blood flow decreases. This level is associated with arrhythmias (due to electrical alterations including ions channel or ion homeostasis disruption) (Roden et al., 2002), does not promote ventricular contractile dysfunction and is reversible on reperfusion (Powers et al., 2007). The second, ischaemic periods of up to 5 to 20 minutes, leads to cardiac injury commonly known as myocardial stunning which, first described by Heyndrickx et al. (1975), refers to a reversible regional depression of myocardial contractility that persists after reperfusion despite the normalisation of blood flow. It is primarily characterized by a reversible contractile dysfunction due to a depressed energy production and an alteration in ion homeostasis without cardiac myocyte death (Roden et al., 2002). The third, ischaemic periods beyond 20 to 30 minutes, leads to irreversible damage characterized by cell death or myocardial infarction (Verma et al., 2002; Skyschally et al., 2008). Previously, necrosis, also termed “oncosis” was regarded as the only mode of cell death, but it is currently known that cells may also die *via* apoptosis and autophagy (Opie et al., 2004). Necrosis is described as an accidental or spontaneous cell death characterized by organelle swelling and membrane rupture, independent of energy supply and caspase cleavage. Apoptosis or programmed cell death type I is characterized by cell shrinkage with DNA fragmentation and membrane blebbing. It is an energy-requiring and caspase-dependent process. Autophagy or programmed cell death type II, differs from apoptosis as it is caspase-independent and morphologically resembles necrosis (for review see Buja & Weerasinghe, 2008; Kang & Izumo, 2003).

The major determinants of the final infarct size (amount of tissue irreversibly damaged) include the size and the location of the perfusion territory distal of the coronary occlusion, the residual blood flow during ischaemia through collaterals (the severity of ischaemia), the temperature, and the hemodynamic situation during ischaemia (Skyschally et al., 2008). The mechanism of infarction will be described later after the description of reperfusion injury.

2.1.2.2. Metabolic and ultrastructural changes associated with ischaemia

It has been observed that ischaemia is associated with ultrastructural alterations characterized by swelling of the sarcoplasmic reticulum, myofibril contracture, and mitochondrial damage including swelling, decreased matrix density and partial loss of cristae (Puri et al., 1975; Jennings & Ganote, 1976; Schaper et al., 1986). These ultrastructural changes are linked to metabolic changes which include depletion of energy stores (Puri et al., 1975); accumulation of metabolic by-products such as lipid metabolites, intracellular acidosis, intracellular calcium (Ca^{2+}) accumulation, reactive oxygen species and increased Na^+ which, together with the loss of K^+ during ischaemia, is largely responsible for the increased cytosolic Ca^{2+} and the induction of mitochondrial dysfunction as well as the impairment of contractile function (Iwai et al., 2002; Murphy & Steenbergen, 2008).

2.1.3. Myocardial reperfusion injury

It has been mentioned that the myocardium can tolerate 15 to 20 minutes of ischaemia (Verma et al., 2002; Skyschally et al., 2008) and the restoration of blood flow may result in arrhythmias, contractile dysfunction, microvascular impairment (endothelial dysfunction) as well as irreversible myocardial damage (infarction) (Kloner, 1993). “Lethal reperfusion injury” refers to the damage caused by reperfusion, resulting in death and loss of cells that were only reversibly injured during the preceding ischaemic episode (Kloner, 1993). It takes place in the first minutes of reperfusion and, although reperfusion limits the progression of ischaemic injury, it worsens ischaemic alterations. Lethal reperfusion injury may contribute up to 50% towards the final size of the myocardial infarct (Yellow & Hausenloy, 2007). I/R-induced contractile dysfunction is mediated by the generation of reactive oxygen/nitrogen species (Bolli et al., 1988 & 1989), a cytosolic calcium overload (Du Toit & Opie, 1992) and an altered contractile protein structure (Kloner et al., 1989).

Other types of myocardial dysfunction are reperfusion arrhythmias (Roden et al., 2002), stunning (Dorge et al., 1998; Bolli & Marbán, 1999) and the no-reflow phenomenon (Ito, 2006) which are beyond the focus of our study. We will only focus on irreversible injury and particularly myocardial infarction.

2.1.4. Mechanism of myocardial infarction

A myocardial infarction is the result of a prolonged ischaemic event as previously mentioned. The major effects of ischaemia are poor oxygen delivery (hypoxia) and poor washout of metabolites including lactate and protons associated with a severe cellular acidosis (low pH). The resultant depressed mitochondrial metabolism and the consequent impairment in glycolysis and fatty acid metabolism lead to a decreased energy production and inhibition of ion pumps with a subsequent alteration in ion homeostasis (increased Ca^{2+} and K^+ loss). In addition, there is membrane damage by fatty acid metabolites and free radicals or as a result of lysosomal activation induced by severe cellular acidosis. All these reactions (leading to proteolysis, contracture and mitochondrial damage) exacerbate the ischaemic insult and culminate in cell death or infarction (Opie, 2004).

It is well established that reperfusion injury is also mediated by excessive oxygen/nitrogen radical generation, increased Ca^{2+} overload, microvascular injury (endothelial dysfunction), and altered myocardial metabolism as well as by the restoration of physiological pH. In addition, inflammation and the opening of mitochondrial permeability transition pore (MPTP) (non selective pore) contribute to reperfusion injury (Buja et al., 2005; Yellow & Hausenloy, 2007). Indeed, during reperfusion, reactive oxygen species (ROS) are generated by xanthine oxidase (mainly from endothelial cells) and the re-energized electron transport chain (ETC) in the cardiomyocyte mitochondria as well as by NADPH oxidase (mainly from neutrophils) (Dhalla et al., 2000a). ROS mediate myocardial injury by: 1) inducing mitochondrial permeability transition pore (MPTP) opening, 2) acting as neutrophil chemo-attractants, 3) mediating dysfunction of the sarcoplasmic reticulum, 4) contributing to intracellular Ca^{2+} overload and 5) damaging essential molecules (lipid, DNA, protein). MPTP opening induces mitochondrial swelling. The rapid washout of lactic acid and metabolites together with the re-energized ETC in the setting of increased intracellular Ca^{2+} results in cardiomyocyte death by hypercontracture (Powers et al., 2007).

2.1.5. New cardioprotective strategies: an opened road

The purpose of the cardioprotective strategies or injury prevention is directed at reducing myocardial I/R injury i.e., the reduction of infarct size and the full restoration of myocardial function. Two accepted endogenous mechanisms are ischaemic preconditioning (IPC) and postconditioning (IPOC), which refer to the cardioprotection obtained by applying short periods

of ischaemia separated by short reperfusion intervals either before the index ischaemia or at the beginning of reperfusion, respectively (Jennings et al., 1991; Murry et al., 1986; Zhao & Vinten-Johansen, 2006). Because of the need to intervene before the index ischaemic event, IPC is clinically not possible. Recently, a better understanding of the mechanisms of IPC and IPOC has opened the road to other possibilities in cardioprotection including remote ischaemic “conditioning” and pharmacological conditioning (for review see Hausenloy & Yellon, 2008). The first refers to short ischaemic periods applied to organs other than the heart (muscle for example) which confers cardioprotection. The second involves the use of pharmacological agents (in place of IPOC or IPC) that are able to activate the same intracellular signalling pathways as do IPC or IPOC. In view of this, research has focused on activation of the reperfusion injury salvage kinase (RISK) pathway which includes protein kinase B (PKB/Akt) and extracellular regulated kinases 42 and 44 (ERK42/44), the inhibition of protein kinase C-delta and opening of mitochondrial permeability transition pore (MPTP) (for reviews see Hausenloy & Yellon (2007), Yellon & Hausenloy (2007) and Hausenloy (2009)).

Nowadays, there is an increasing need for effective cardioprotective strategies that are able to improve the clinical outcomes of patients with ischaemic heart disease, which is the worldwide number one killer (Lopez et al., 2006). In this regard, we have recognized that I/R injury is influenced by cardiovascular risk factors (e.g., diabetes, obesity, hyperlipidaemia, hypercholesterolemia, ageing) which may favour or abolish cardioprotection induced by pre or post-conditioning (Ferdinandy et al., 2007; Balakumar et al., 2009). Thus, from our understanding of pre and postconditioning, the road has been opened to cardioprotection; however, an ideal strategy must retain its efficacy even under pathological conditions. This means that it must not only be effective in lean normal subjects but also in obese or diseased subjects. To gain more insight into cardioprotection in obesity, the impact of obesity on cardiovascular disease is reviewed in the following section. The effects of obesity on I/R injury in particular are discussed in obesity-related cardiac alterations (section 2.2.3).

2.2. OBESITY AND CARDIOVASCULAR DISEASE

2.2.1. Introduction

Obesity is defined as a chronic metabolic disorder characterized by an increased amount of body fat to the extent that adverse health consequences may occur. Several epidemiological and animal studies have established a strong association between obesity and the development of cardiovascular disorders including coronary heart disease, heart failure and sudden death (Zalesin et al., 2008; Poirier et al., 2006; Abel., 2008; Harmancey et al., 2008; Wong et al., 2007; Chess & Stanley, 2008). The link between obesity and cardiovascular disease and the mechanism underlying the development of diet-induced metabolic abnormalities still remain poorly elucidated. Increased fat accumulation and adipose tissue-derived hormone abnormalities, lipotoxicity with increased oxidative stress and insulin resistance, have been suggested to be the possible mechanisms linking obesity to its cardiovascular complications (Van Gaal et al., 2006; Grattagliano et al., 2008; Cornier et al., 2008; Chess & Stanley, 2008; Savage et al., 2007). Thus, this section discusses the obesity-induced metabolic alterations that may contribute to cardiac alterations. Vascular alterations as well as atherosclerosis, hypertension and other obesity-related disorders are beyond the focus of our study (for review see Poirier et al., 2006).

2.2.2. Obesity-induced systemic metabolic alterations

2.2.2.1. Metabolic syndrome (MS)

Obesity, particularly central obesity, leads to a cluster of metabolic abnormalities associated with increased cardiovascular disease risk (Eckel et al., 2005). This clustering has been termed the metabolic syndrome (MS), a concept proposed and published by the WHO and other medical groups including the National Cholesterol Education Program's Adult Treatment Panel III (NCEP ATP III) for an easy clinical diagnosis and treatment of an increased cardiometabolic risks (Cornier et al., 2008). Metabolic syndrome components include visceral obesity, insulin resistance, glucose intolerance, atherogenic dyslipidaemia, raised blood pressure, and a pro-inflammatory state (Opie, 2007). The concept of metabolic syndrome has been the subject of debate regarding its definition and utility (Cornier et al., 2008; Raeven,

2004). Indeed, beside cardiovascular disease as the primary clinical outcome of the metabolic syndrome, it has been demonstrated that metabolic syndrome is also associated with other pathological conditions such as type II diabetes mellitus, non alcoholic fatty liver disease, reproductive disorders and some cancers (Pothiwala et al., 2009). Therefore, the concept of metabolic syndrome must be considered as an evolving concept and its definition could be extended to its new additional clinical components (Cornier et al., 2008; Huang, 2009).

The pathophysiology of metabolic syndrome has been largely reviewed (Cornier et al., 2008; Eckel et al., 2005; Grundy, 2004; Pothiwala et al., 2009; Steinberger et al., 2009). Its progressive development has been represented (see fig.2.1) as a result of the intersection between obesity and insulin resistance which is characterized by abdominal obesity, abnormal circulating lipids, and high blood pressure and glucose (Steinberger et al., 2009). From this intersection emerges an increase in visceral fat, oxidative stress, inflammation, adipocytokines dysfunction, and vascular abnormalities, and increased circulating cortisol; a hallmark of cardiovascular disease and type 2 diabetes mellitus (Steinberger et al., 2009).

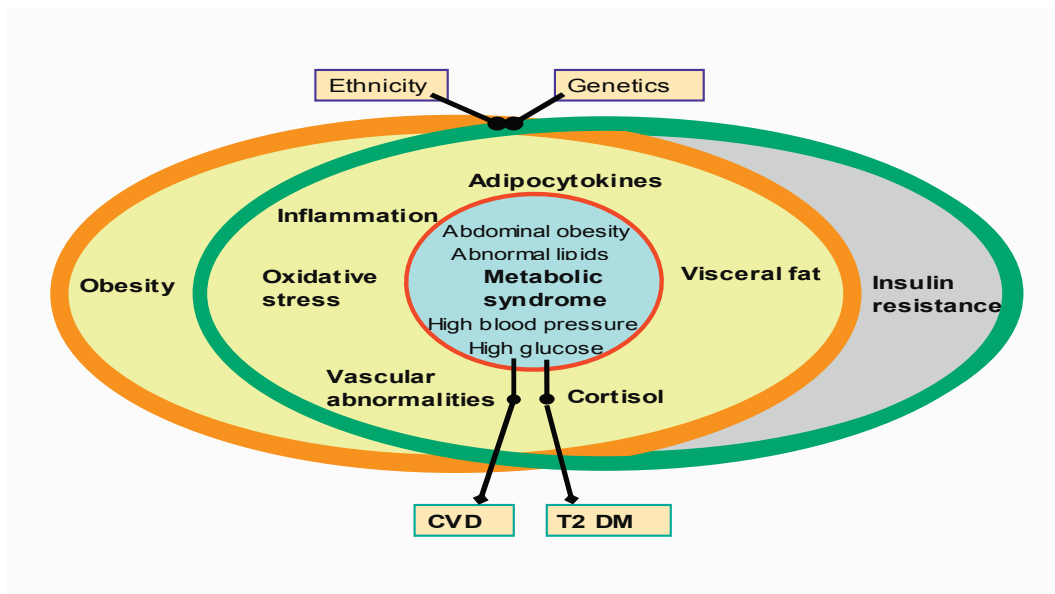


Figure 2- 1 Schematic representation of components of the metabolic syndrome (MS)
CVD, cardiovascular disease; T2DM, type 2 diabetes mellitus (adapted from Steinberger et al., 2009).

Obesity has been referred to as the only central and reversible cardiovascular risk factor that favourably influences all other associated cardiovascular risk markers including high-density lipoprotein cholesterol, high-sensitivity C-reactive protein, hypertension, low-density lipoprotein cholesterol, triglycerides and renin-angiotensin-aldosterone system/sympathetic nervous system (Zalesin et al., 2008). In addition, the expression of these metabolic conditions has

been found to be the result of complex interactions between genetics or ethnicity and environmental factors including life style (physical activity, dietary intake, television-watching habits) (Zalesin et al., 2008; Steinberger et al., 2009). Consequently, the increasing obesity pandemic is the driving force behind the actual rising prevalence of the metabolic syndrome where increased adiposity and subsequent metabolic alterations play an important role (Grundy, 2008).

2.2.2.2. Role of adipose tissue in diet-induced metabolic alterations

The metabolic syndrome by definition has been associated with increased adiposity (Grundy, 2008). It is now known that adipose tissue plays a crucial role in normal and pathological processes in the body (Vazquez-Vela et al., 2008). Under normal conditions, adipose tissue acts as a store of the surplus energy during increased food intake or reduced energy expenditure (Sethi & Vidal-Puig, 2007). The surplus energy is deposited in adipose tissue in the form of neutral triglycerides (Sethi & Vidal-Puig, 2007). As a consequence, circulating free fatty acids (FFA) increase and excessive fats accumulate inappropriately in non-adipose tissues, such as liver and muscles, including the heart, and negatively affect their normal metabolism and function (Christoffersen et al., 2003; Shimabukuro, 2009). This situation has been referred to as steatosis-induced lipotoxicity and plays an important role in cardiac alterations induced by obesity (Banerjee & Peterson, 2007).

Beside its role as energy store, it is now recognized that adipose tissue also behaves as a secretory and endocrine organ releasing a range of bioactive substances into the circulation (Kershaw & Flier, 2004). These substances have both local (autocrine and/or paracrine) and systemic (endocrine) actions in the regulation of a variety of physiological/metabolic processes including adipocyte differentiation, local and systemic inflammation, overall energy balance, blood pressure, and glucose and lipid metabolism (Ahima & Osei, 2008; Matsuzawa, 2006). The role of adipose tissue as a secretory organ will be summarized in the following section.

2.2.2.2.1. Adipose tissue-derived secretion

Adipose tissue secretes a range of bioactive substances including adipocytokines (also called adipokines) such as leptin, adiponectin, resistin, visfatin, apelin, omentin, and chemerin

(Kershaw & Flier, 2004; Vazquez-Vela et al., 2008; Matsuzawa, 2006). Others that are also secreted by other tissues include tumor necrosis factor α (TNF- α), interleukin 6 (IL-6), monocyte chemo-attractant protein 1 (MCP-1), lipoprotein lipase, plasminogen activator inhibitor 1 (PAI-1), and angiotensinogen (AT) (Vazquez-Vela et al., 2008).

Obesity results in dramatic alterations in the release of bioactive substances by adipose tissue (see fig.2.2). Compared to normal subjects, circulating leptin levels are elevated in obesity (Lin et al., 2000) and contrary to expectations, circulating adiponectin levels are decreased in obese subjects despite being produced in adipose tissue (Arita et al., 1999). Similar to leptin, apelin, angiotensin II (ATII), TNF- α and IL-6 are elevated in obesity (Du Toit et al., 2008; Maury et al., 2009).

Adipocytokines are active in a range of processes, such as the control of nutritional intake (leptin), insulin sensitivity and inflammatory processes (TNF- α , IL-6, resistin, visfatin, adiponectin) (Vazquez-Vela et al., 2008). Adipocytokines have been viewed as a bridge connecting obesity and insulin resistance (for review see Zhuang et al., 2009) and are implicated in cardiovascular function (for review see Gualillo et al., 2007). Though other adipose tissue-derived secretions have been shown to play an important role in systemic metabolic alterations associated with obesity (Qatanani & Lazar, 2007; Vazquez-Vela et al., 2008), only the role of leptin and adiponectin will be briefly discussed in this section.

2.2.2.2.1.1. Leptin

Leptin is a non glycosylated peptide hormone mainly produced by adipocytes (Zhang et al., 1994). It is encoded by the obese (*ob*) gene and plays an important role in food intake and body weight gain regulation in diet-induced obesity (Lin et al., 2000). Leptin has anorexic properties and acts as regulator factor in the hypothalamus inducing a reduction in food intake and an increase in energy expenditure. In peripheral tissues, leptin appears to prevent fat deposition in non-adipose tissue, thus enhancing insulin sensitivity in muscle and fat and preventing “lipotoxicity” of pancreatic β -cells (Zhuang et al., 2009). At a certain stage of food intake leptin actions become defective and leptin resistance occurs (Lin et al., 2000).

Using the mouse model of diet-induced obesity, Lin et al. (2000) have shown that increased body weight gain was associated with an elevation in circulating leptin levels.

In this study, during 19 weeks of feeding, they found that in the early stage of obesity (from one week) leptin levels were increased and mice responded to exogenous leptin injection. At the middle stage (from eight weeks onwards), the increase in leptin levels was accompanied by a reduction in food intake and a gradual loss of leptin sensitivity (i.e., variable with the dose of leptin injected in the test). At the later stage (from fifteen weeks), they found that there was an increase in food intake associated with a loss in leptin sensitivity. Although the mechanism causing central leptin insensitivity is not fully understood, it has been suggested that the presence of high levels of leptin may induce desensitization of the hypothalamic leptin receptor by down-regulation of leptin receptor density or even saturation of the receptors with endogenous leptin as a result of the elevated leptin output (Widdowson et al., 1997).

Elevated serum leptin levels are associated with hyperinsulinaemia and correlate positively with visceral fat accumulation (Kim-Motoyama et al., 1997). Leptin and insulin (secreted by pancreatic β -cells) are considered as peripheral adiposity signals to the central nervous system in the control of food intake and metabolism where the secretion of insulin increases with meals and circulating nutrients (Benoit et al., 2004). During the development of diet-induced obesity, there is a crosstalk between leptin and insulin signalling (Morrison et al., 2009). Leptin can modulate insulin sensitivity and/or be induced by insulin (Morrison et al., 2009). Hyperinsulinaemia is associated with hyperleptinaemia and a compensatory reduction in leptin sensitivity (leptin resistance) through dysregulation of the adipose tissue-hypothalamic axis (Almanza-Perez et al., 2008; Anubhuti & Arora, 2008; Benoit et al., 2004). Leptin resistance is reversible. It has recently been shown that weight loss by prolonged calorie restriction was accompanied with a reduction in leptin levels and improvement of obesity and its related metabolic abnormalities (Hammer et al., 2008).

With regard to its cardiovascular effects, there is a strong association between leptin resistance and the activation of the sympathetic nervous system, prothrombotic effects, endothelial dysfunction, vascular smooth muscle hypertrophy, myocardial remodelling, heart failure, atherosclerosis and hypertension, and other related metabolic disorders (Gualillo et al., 2007; Lago et al., 2008).

2.2.2.2.1.2. Adiponectin

Adiponectin has been shown to be the link between obesity and its cardiovascular complications (Shibata et al., 2009). It has antiatherogenic (Lara-Castro et al., 2007), antidiabetic and anti-inflammatory properties (Matsuzawa, 2006). Plasma adiponectin concentrations have been found to correlate negatively with waist circumference, visceral fat area, serum triglyceride concentration, fasting plasma glucose, fasting plasma insulin, and systolic and diastolic blood pressure (Ryo et al., 2004). Adiponectin administration increases fatty acid oxidation in the muscle and potentiates insulin-mediated inhibition of hepatic gluconeogenesis (Matsuzawa, 2006). It has also been shown to be cardioprotective. Administration of adiponectin protected the heart against ischaemia/reperfusion injury by reducing myocardial infarct size through activation of 5'AMP-activated protein kinase (AMPK) and by the suppression of cardiac production of TNF- α through activation of the cyclooxygenase-2-prostaglandin-E2 (COX-2-PGE2) pathway (Shibata et al., 2005). A recent study by Gonon et al. (2008) demonstrated that adiponectin protects against myocardial ischaemia/reperfusion injury *via* activation of AMPK, protein kinase B (PKB)/Akt and nitric oxide. Because adiponectin is downregulated in obese subjects (Arita et al., 1999), its circulating levels have been identified as the clinical marker of cardiometabolic diseases (Matsuzawa, 2006).

In cardiovascular disease, downregulation of circulating adiponectin (hypo adiponectinaemia) has been associated with increased visceral fat accumulation, hyperlipidaemia, endothelial dysfunction, hyperglycaemia, inflammatory atherosclerosis, increased coronary heart disease risk and hypertrophy (Gualillo et al., 2007). The mechanism of this downregulation is not yet fully understood. The downregulation of adiponectin is associated with elevated tumor necrosis factor- α (TNF- α) levels in obesity. Incubation of human visceral adipose tissue from patients without diabetes mellitus with TNF- α (5.75 nmol/l), has shown a decrease in adiponectin mRNA expression of 97% (Hector et al., 2007). This could explain the association of elevated TNF- α secretion with reduced levels of adiponectin seen in obese subjects (Arita et al., 1999).

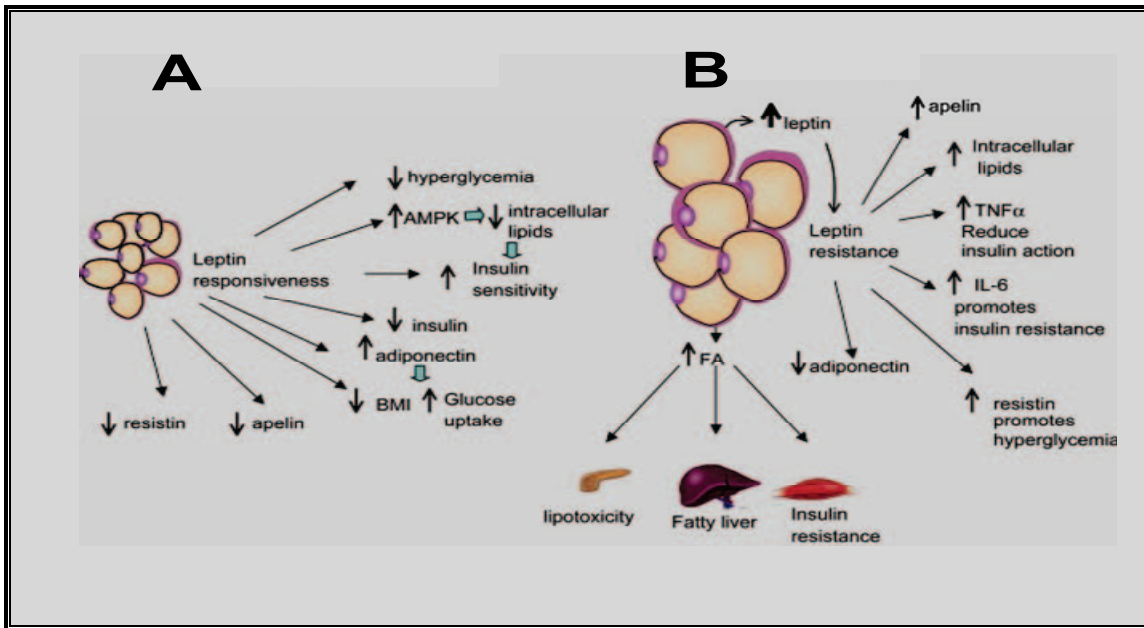


Figure 2- 2 Obesity and adipocyte response (Vazquez-Vela et al., 2008)

Adipose tissue-derived proteins secretion during energy equilibrium (A) and obesity (B)

During energy equilibrium without obesity, adipocytes are leptin responsive and non-hypertrophic and, therefore, non-adipose tissues are leptin and insulin sensitive. Under these conditions adipocytes secrete adipocytokines (leptin and adiponectin) to stimulate insulin sensitivity and fatty acids (FA) oxidation with increased AMPK activation. This is characterized by increased glucose uptake, reduced circulating insulin and glucose levels. At the same time, adipocytokines that promote insulin resistance (apelin and resistin) are reduced. During obesity, adipocytes are hypertrophic and non-adipose tissues become resistant to leptin and insulin action. Adipocytes secrete high amounts of FA as well as adipocytokines that promote insulin resistance resulting in ectopic accumulation of lipids (lipotoxicity) in pancreas, liver and skeletal muscle. TNF- α , IL-6, apelin and resistin secretion increases while adiponectin decreases. This is associated with elevated leptin production and leptin resistance (Vazquez-Vela et al., 2008).

2.2.2.3. Obesity-induced dyslipidaemia

It is well established that obesity is associated with circulating lipid abnormalities or dyslipidaemia (Franssen et al., 2008). This entails dysregulation of lipid metabolism involving several different processes (lipoprotein hydrolysis, fatty acids uptake, synthesis, and esterification) leading to an increase in serum triglyceride and free fatty acids (FFA) levels combined with the deposition of triglyceride in non-adipose tissue (Franssen et al., 2008). The term “Atherogenic lipoprotein phenotype” or “lipid triad” is used to describe a common form of

dyslipidaemia, characterized by three lipid abnormalities. These include increased plasma triglyceride levels, decreased high density lipoprotein- cholesterol (HDL-c) concentrations and the presence of small dense low density lipoproteins (sdLDL) particles (Bamba & Rader, 2007). Atherogenic dyslipidaemia is one of the important elements used for the diagnosis of metabolic syndrome (Grundy et al., 2005). It has been described as an early feature of the metabolic syndrome and frequently precedes glucose intolerance (Reaven, 1988).

The mechanism of development of dyslipidaemia has been intensively reviewed (Chan et al., 2004; Vinik, 2005, Howard et al., 2003). Dyslipidaemia results from the action of hyperinsulinaemia on lipoprotein metabolism (Avramoglu et al., 2006). The reduction in high density lipoprotein-cholesterol (HDL-c) may result from a reduction in flux of apolipoproteins and phospholipids from chylomicrons and very low density lipoproteins (VLDL) particles, which are normally used in HDL-c maturation (Franssen et al., 2008). In addition, an increase in circulating FFA and hepatic insulin resistance, accompanied by increased apolipoprotein-B (Apo-B), induce the assembly and secretion of VLDL by the liver leading to an overall increase in serum triglyceride or hypertriglyceridaemia and a reduced HDL-c (Avramoglu et al., 2006).

Although an increase in low-density lipoprotein cholesterol (LDL-c) levels is a cardiovascular risk factor, it is not a component of the metabolic syndrome (Grundy, 2008). In fact, structural changes occur in LDL-cholesterol particles, which become smaller, dense and more atherogenic. The obesity-induced atherogenic dyslipidemia has been implicated in the genesis of atherosclerosis (Mittendorfer et al., 2008; Libby, 2002). Hence, the increased risk of coronary heart disease in obesity is in part due to its strong association with atherogenic dyslipidaemia (Bamba & Rader, 2007).

2.2.2.4. Obesity- induced insulin resistance

2.2.2.4.1. Concept of insulin resistance

Insulin is a pleiotropic hormone produced and released into circulation by pancreatic β -cells with effects on metabolism and various cellular processes in different tissues of the body including adipose tissue, liver and muscle (Jellinger, 2007).

Insulin stimulates glucose uptake in skeletal muscle and the heart, suppresses the production of glucose and very low density lipoprotein (VLDL) in the liver, and inhibits FFA release from adipose tissue and synthesis of proteins from amino acids (Kahn & Flier, 2000). The concept of insulin resistance refers to the condition in which the body produces insulin but does not respond to it properly because of a decreased cellular sensitivity to its effects on glucose uptake, metabolism and storage (Muniyappa et al., 2008).

Based upon the relationship between insulin resistance, hyperglycaemia, dyslipidaemia, and hypertension as mediators for cardiovascular disease, Reaven created the concept of syndrome X or insulin resistance syndrome as a cluster of common metabolic abnormalities and clinical outcomes associated with insulin resistance (Reaven, 1988). Insulin resistance is associated with elevated circulating fasting insulin levels (Du Toit et al., 2008; Kim & Reaven, 2008). It can be measured by various methods including amongst others fasting plasma or serum insulin concentration, quantitative insulin sensitivity check index (QUICK index), and homeostasis model assessment (HOMA) index (Muniyappa et al., 2008). The HOMA index is calculated as the product of fasting insulin (mU/L) and glucose concentration (mmol/L) divided by 22.5. To gain more insight into the phenomenon of insulin resistance in obesity and its possible interaction with melatonin treatment, its mechanism of action and signalling are summarized below.

2.2.2.4.2. Mechanisms of obesity-induced insulin resistance

It is well established that high fat/calorie feeding causes obesity and the subsequent development of insulin resistance (DeFronzo & Ferrannini, 1991; Haag & Dippenaar, 2005). It has been demonstrated that rats with diet-induced obesity developed insulin resistance and dyslipidaemia with (Lima-Leopoldo et al., 2008) or without (Du Toit et al., 2008) glucose intolerance. This insulin resistance was accompanied by hyperleptinaemia (Dourmashkin et al., 2005). It could also be developed in mouse (Lin et al., 2000; Thakker et al., 2006), and rabbit (Zhao et al., 2008) models of diet-induced obesity.

Obesity is the most important factor in the aetiology of insulin resistance (Cornier et al., 2008; Reaven, 1988). Genetic and ethnic predisposition including family history, increased circulating free fatty acids (FFA) and tumor necrosis factor- α (TNF- α) associated with the dysregulation of adipose tissue-derived secretion (see fig.2.2) have been mentioned as factors that

influence the development of insulin resistance (Mlinar et al., 2007). In particular, elevated circulating free fatty acids in obesity are identified as the driving force behind insulin resistance (Eckel et al., 2005). However, not all overweight/obese persons develop insulin resistance and a normal weight does not equate to insulin sensitivity. For example, the study done by McLaughlin et al. (2004) has found that among people who developed insulin resistance 36% were obese (BMI ≥ 30.0 kg/m²), 30% overweight or obese (BMI ≥ 25 kg/m²) while 16% were of normal weight (BMI ≤ 25 kg/m²).

The mechanisms of obesity induced insulin resistance are presented in fig.2.3 and 2.4. Briefly, in obesity, elevated tumour necrosis factor-alpha (TNF- α) induces the release of free fatty acids (FFA) from adipose tissue into the circulation. In the liver, the deposition of FFA with the action of apolipoprotein-B, results in increased accumulation of triglyceride (TRIG) and an increased secretion of very low density lipoprotein (VLDL). Together with this there is a decrease in serum high density lipoprotein-cholesterol (HDL-C) and an increase in low density lipoprotein-cholesterol (LDL-C) accompanied by gluconeogenesis. In muscle, FFA and TNF- α inhibit insulin-mediated glucose uptake pathways which result in a reduced insulin sensitivity contributing to hyperglycaemia. Accumulation of excessive FFA as triglyceride droplets in muscle and other non-adipose tissues can induce excessive reactive oxygen species production and increase oxidative stress which is also implicated in the pathogenesis of insulin resistance (Grattagliano et al., 2008). The obesity-induced oxidative stress is discussed in section 2.2.2.5.

Other factors such adipocytokines have been implicated as the link between increased adipose tissue accumulation and insulin resistance (Zhuang et al., 2009). Adiponectin improves insulin sensitivity favouring glucose and fatty acid oxidation in muscle. In liver, it induces fatty acid oxidation, decreases gluconeogenesis and lipid synthesis. Adiponectin levels increase with weight loss and decreases with insulin resistance. The role of leptin has previously been reviewed in section 2.2.2.2. In the early stage of diet-induced increased body weight, leptin increases insulin sensitivity by decreasing fat deposition in peripheral tissues. At a later stage, long-term increases in circulating leptin lead to a reduced role of leptin and the development of insulin resistance. Although other adipocytokines also play a crucial role in obesity-induced insulin resistance (Maury et al., 2010; Antuna-Puente et al., 2008; Trujillo & Scherer, 2006), the description of their involvement is beyond the focus of our study.

The exact mechanism of insulin resistance is however, not completely understood. Glucocorticoid hormones associated with high circulating FFA contribute to the impairment of insulin actions by various mechanisms (Kennedy et al., 1993; Mlinar et al., 2007).

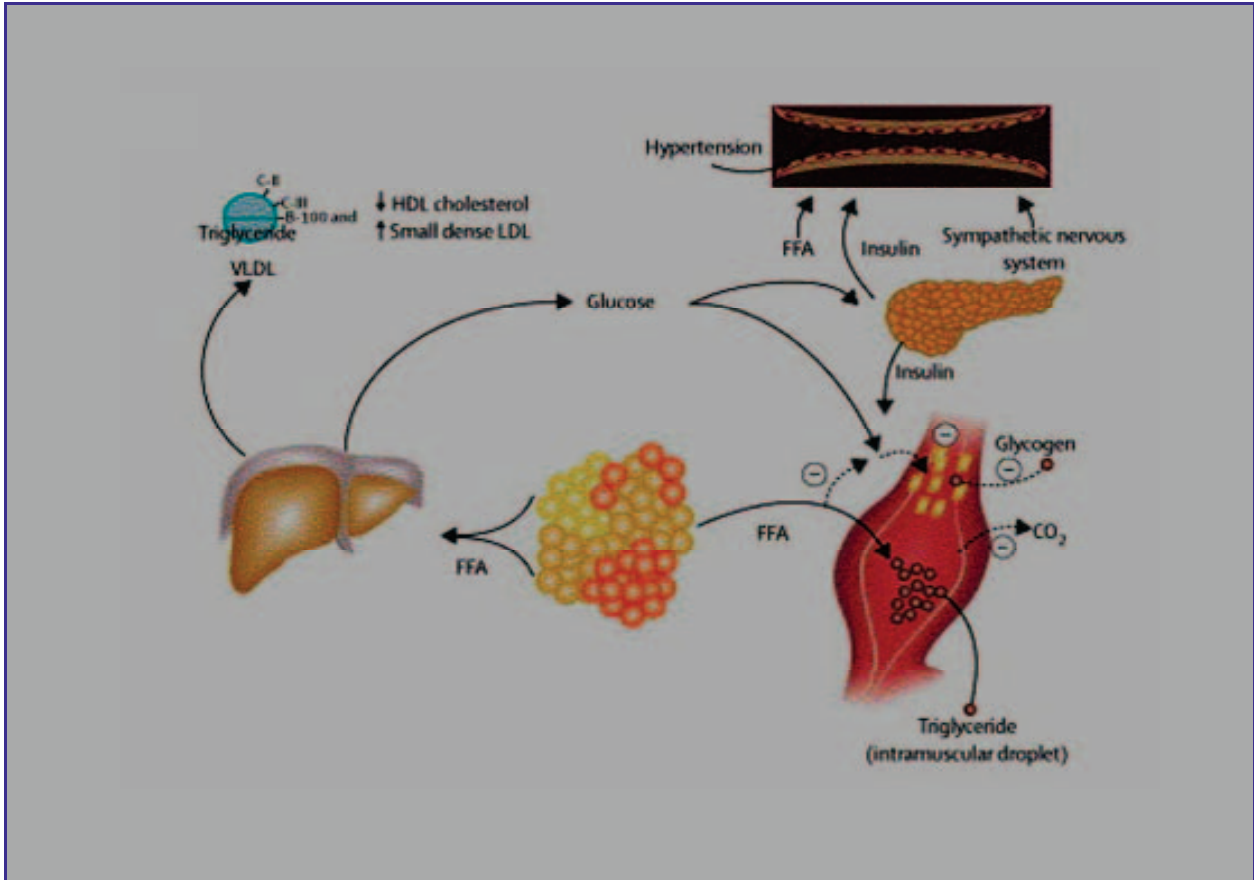


Figure 2- 3 Mechanisms implicated in FFA induced-insulin resistance.

Free fatty acids (FFA) are released in abundance from an expanded adipose tissue mass. In the liver, FFA increase production of glucose, triglycerides and secretion of very low density lipoproteins (VLDL). Associated lipid/lipoprotein abnormalities include reduction in high density lipoprotein (HDL) cholesterol and increased low density lipoproteins (LDL). FFA also reduce insulin sensitivity in muscle by inhibiting insulin-mediated glucose uptake. Associated defects include a reduction in glucose partitioning to glycogen and increased lipid accumulation in triglyceride (TRIG). Increases in circulating glucose and to some extent FFA, increase pancreatic insulin secretion resulting in hyperinsulinaemia. Hyperinsulinaemia may result in enhanced sodium reabsorption and increased sympathetic nervous system (SNS) activity and contribute to hypertension (adapted from Eckel et al., 2005).

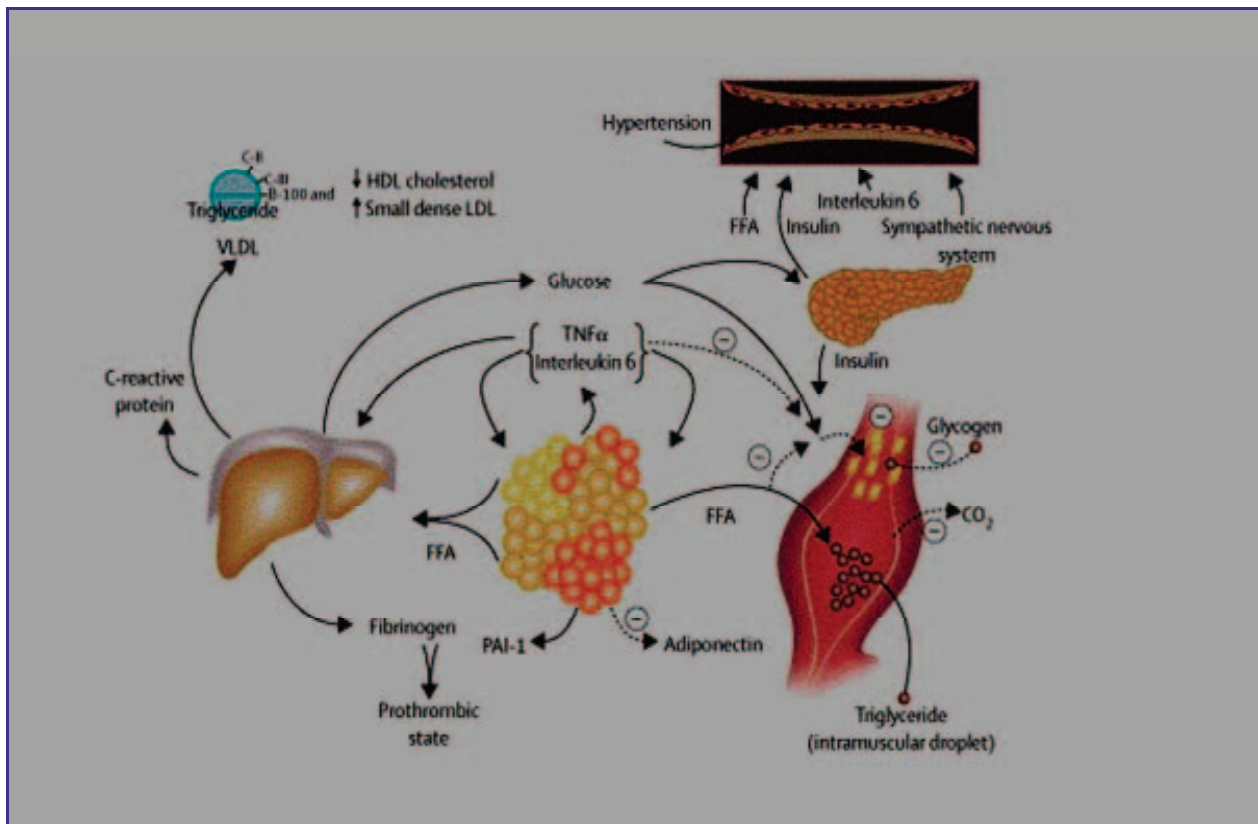


Figure 2- 4 Other factors implicated in mechanism of insulin resistance.

Superimposed and contributory to the insulin resistance produced by excessive FFA are the paracrine and endocrine effect of the proinflammatory state. Produced by a variety of cells in adipose tissue including adipocytes and monocyte-derived macrophages, the enhanced secretion of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF- α) among others results in more insulin resistance and lipolysis of adipose tissue triglyceride stores to circulating FFA. IL-6 and other cytokines are increased in the circulation and may enhance hepatic glucose production, the production of very low density lipoproteins (VLDL) by the liver and insulin resistance in muscle. Cytokines and FFA also increase the production of fibrinogen and plasminogen activator inhibitor-1 (PAI-1) by the liver that complements the overproduction of PAI-1 by adipose tissue. This results in a pro-thrombotic state. Reductions in the production of the anti-inflammatory and insulin sensitizing cytokine adiponectin are also associated with the metabolic syndrome and may contribute to the pathophysiology of the syndrome. FFA: free fatty acids (adapted from Eckel et al., 2005)

2.2.2.4.3. Insulin signalling in obesity

2.2.2.4.3.1. Insulin signalling in sensitive tissues (fig.2.5)

To confer its action, insulin binds to its receptors (IR) in the cell membrane and causes its autophosphorylation creating docking sites for downstream interacting proteins such as insulin receptor substrate 1 to 4 (IRS1-4) from where three potential signal transduction pathways may occur (see fig.2. 5): 1) the phosphatidylinositol-3 kinase (PI-3K) dependent pathway, 2) the mitogen-activated protein kinase (MAPK) pathway and 3) the CAP/Cbl/TC10 pathway (Saltiel & Kahn, 2001). The PI-3K dependent pathway is initiated by tyrosine phosphorylation of insulin receptor substrate (IRS-1-4) which, when phosphorylated, associates with the p85, subunit of PI-3K to produce phosphatidylinositol-3, 4, 5-phosphate (PIP₃). Increased PIP₃ results in activation of protein kinase B (PKB/Akt) *via* activation of phosphoinositide-dependent kinase 1 (PDK1) and other down stream effector molecules which mediate the metabolic effects of insulin including translocation of glucose transporter 4 (GLUT-4) to the membrane, and glycogen synthesis *via* PKB/Akt mediated inhibitory phosphorylation of glycogen synthase kinase 3 (GSK-3) (Shulman et al.,1999). In parallel with PKB/Akt, PDK1 is also able to activate the atypical protein kinase C (PKC) ζ and λ (see fig.2.5). This insulin-induced activation of PKC ζ and λ has however not yet been demonstrated in heart tissue (Bertrand et al., 2008). The MAPK-dependent pathway is initiated by the successive activation of Shc, Grb₂, mSOS, and Ras. Ras activation triggers the cascade of Raf to MEK₁ to extracellular regulated kinase (ERK) 42 and 44. ERK 42 and 44 are MAPK subtypes that mediate the mitogenic and the pro-inflammatory effects of insulin (Sasaoka et al., 1994). The third pathway (CAP/Cbl) has been suggested for glucose uptake in order to be fully manifested (Saltiel & Kahn, 2001). In this pathway, from the phosphorylation of insulin receptor substrate (IRS), the adapter protein CAP recruits proto-oncogen Cbl to the phosphorylated IRS and the activation of Cbl results in a cascade reinforcing translocation of GLUT-4 also stimulated by PI-3K pathway but has not yet been established in cardiac tissue (Bertrand et al., 2008). These pathways act in a concerted fashion to coordinate the regulation of vesicle trafficking, protein synthesis, enzyme activation and inactivation, and gene expression, which result in the regulation of glucose, lipid and protein metabolism (see fig. 2.5).

2.2.2.4.3.2. Compromised insulin signalling

Defects in insulin signalling may occur at several levels: pre-receptor (abnormal insulin), receptor (decreased receptor number or affinity), glucose transporters (decreased Glut-4 expression), or post-receptor (abnormal signal transduction and phosphorylation). Among these defects, post-receptor insulin signalling abnormalities contribute significantly to insulin resistance and are associated with various clinical consequences including among others hyperglycaemia-induced tissue damage, dyslipidaemia, inflammation, hypertension, and metabolic syndrome (Reaven et al., 2004). As previously discussed, a major contributor to the development of insulin resistance is excess circulating fatty acids. The molecular mechanism underlying defective insulin-stimulated glucose transport activity can be attributed to increases in intracellular lipid metabolites such as fatty acyl-CoA and diacyl-glycerol, which in turn activate PKC- ϵ leading to defects in insulin signalling through Ser/Thr phosphorylation of insulin receptor substrate (IRS)-1 and a subsequent inhibition of PI-3 K and PKB/Akt (Morino et al., 2006; Chess & Stanley, 2008).

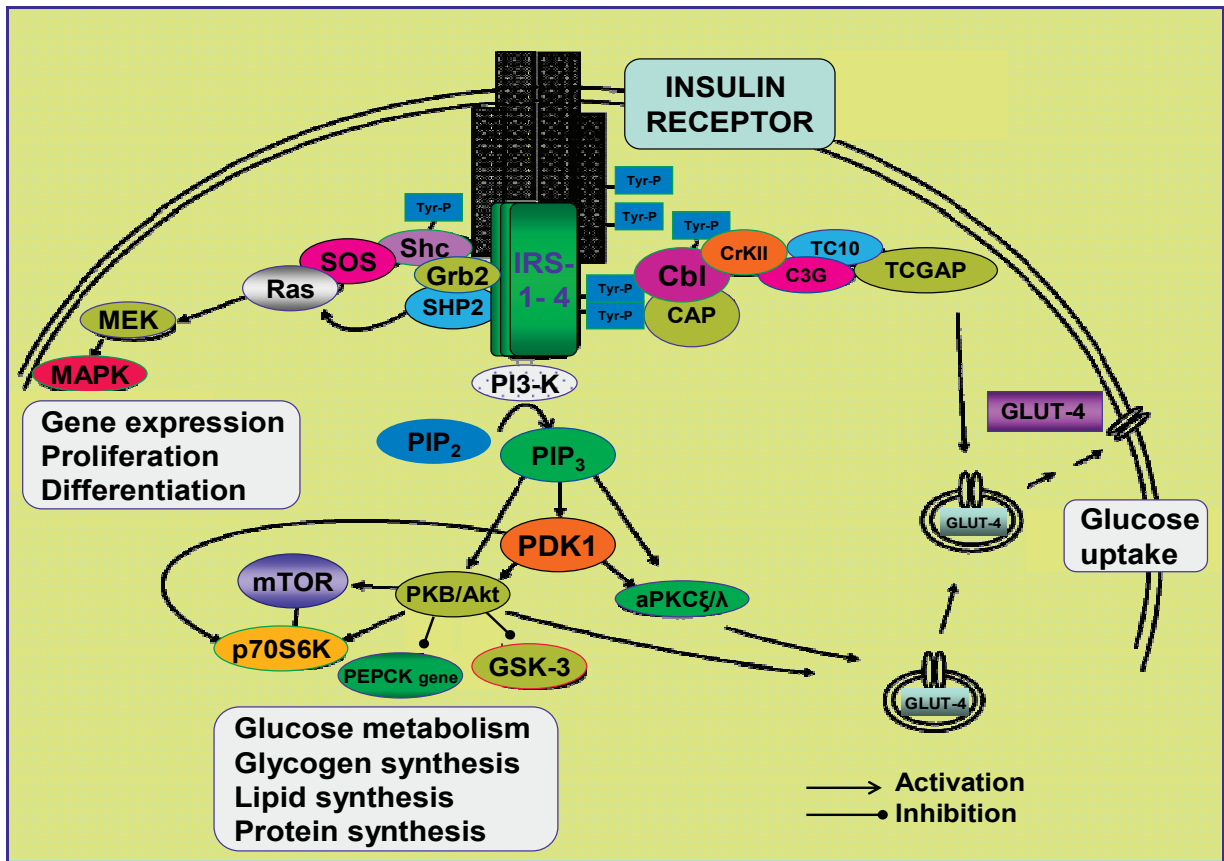


Figure 2- 5 Principal components of the insulin signalling pathways.

Fig.2.5: *Activation of insulin receptor in the cell membrane by insulin causes its autophosphorylation, creating docking sites for downstream interacting proteins such as insulin receptor substrate 1 to 4 (IRS1-4). These are also autophosphorylated providing further docking sites from where three potential signal transduction pathways occur: PI3K-dependent pathway for metabolism (glucose metabolism, glycogen synthesis and protein synthesis), CAP/Cbl pathway (additional pathways required for glucose transporter-4 (GLUT4) translocation), and the mitogen-activated protein kinase (MAPK) pathway (for gene expression, proliferation, differentiation). IRS: insulin receptor substrate; PI3K: phosphoinositide-3-kinase; PDK-1: phosphoinositide-dependent kinase 1; PKB/Akt: protein kinase B; aPKC: atypical protein kinases C; mTOR: mammalian target of rapamycin; p70S6K: p70 ribosomal S6 kinase; PEPCCK: phosphoenol pyruvate carboxykinase; GSK-3: glycogen synthase kinase 3 (Mlinar et al, 2007; Saltiel & Kahn, 2001).*

2.2.2.5. Oxidative stress and obesity

2.2.2.5.1. Oxidative stress

Obesity and its related metabolic alterations are associated with increased oxidative stress (Grattagliano et al., 2008; Keaney et al., 2003; Mittal & Kant, 2009; Urakawa et al., 2003; Vincent et al., 1999; Vincent et al., 2001). Oxidative stress occurs when there is an imbalance between tissue free radicals and reactive oxygen/nitrogen species (ROS/RNS) levels and antioxidant capacity. Under these conditions, free radicals or reactive oxygen/nitrogen compounds accumulate intracellularly and cause damage because of insufficient antioxidant defense capacity against their production and action (Grattagliano et al., 2008; Hansel et al., 2004; Singal et al., 1998). Reactive oxygen species are chemical entities composed of free radicals and non-radical derivatives. Reactive oxygen species (ROS) include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCL), superoxide ($\cdot O_2^-$), hydroxyl ($\cdot OH$), peroxy ($\cdot RO_2^-$) and hydroperoxyl (HO_2^-) radicals. Major reactive nitrogen species (RNS) are nitric oxide (NO) and peroxynitrite ($ONOO^-$) (Evans et al., 2005).

In well balanced conditions, free radicals play a role in the regulation of cell function and may also act as intracellular and extracellular/intercellular signalling molecules. In the unbalanced state, they have been implicated in the aetiology and pathology of many pathological conditions including obesity and its complications (Minoguchi et al., 2006; Roberts & Sindhu,

2009). Excessive free radicals and ROS production can directly affect organs and tissues and cause cell injury by damaging DNA, proteins, and carbohydrate and lipid constituents leading to cell death or apoptosis (Li et al., 2005).

Oxidative stress in tissue is measured by the determination of the levels of lipid peroxidation markers including thiobarbituric reactive acid substances (TBARS), malondialdehyde (MDA), lipid hydroperoxides (PEROX), F₂-isoprostanes (8-epiPGF₂α), and conjugated dienes (CD). It has been shown that lipid peroxidation is increased in obesity and has been associated with a low systemic antioxidant defense (Van Gaal et al., 1998; Vincent et al., 2005; Furukawa et al., 2004; Ozata et al., 2002). Therefore, the levels of major dietary antioxidants including vitamin C (ascorbate), vitamin A, vitamin E, α-lipoic acid and trace minerals (zinc, selenium, and magnesium) can also indicate the oxidative stress levels. They have been found to be lowered in obesity (Singh et al., 1998). Other measurements such as the levels of cellular enzymatic and non enzymatic antioxidants including superoxide dismutase (SOD) and its cytosolic (Cu-ZnSOD) and mitochondrial (Mn-SOD) forms, catalase (CAT), glutathione peroxide (GPx), glutathione reductase (GRd), and glutathione (GSH) content, have also been used as oxidative markers. The levels of these antioxidants are often reduced in obesity (for review see Vincent et al., 2007).

2.2.2.5.2. Obesity-induced oxidative stress

Obesity is associated with elevated oxidative stress and reduced antioxidant content compared to the non obese individuals (Mittal & Kant, 2009). Obesity has been shown to be associated with elevated lipid peroxidation in adult men (Ozata et al., 2002), women (Furukawa et al., 2004), and premenopausal women (Van Gaal et al., 1998) and children (Olusi et al., 2002). In addition, it was associated with low systemic antioxidant defense such as low content of antioxidant enzymes, tissue dietary antioxidants and glutathione (Vincent et al., 2007). This has also been reported by Roberts et al. (2006) showing that chronic consumption of high fat/high refined sugars (sucrose and glucose) results in an elevation in oxidative stress by upregulation of superoxide generating NADPH oxidase and a significant reduction in mitochondrial, and cytoplasmic SOD (Mn SOD, CuZn-SOD, respectively).

Oxidative stress is enhanced as body weight increases. In a study evaluating the metabolic profile of 148 clinically healthy middle-aged women, Wu et al. (2009) found that anthropometric

measurements (body mass index, waist circumference, both central and peripheral fat mass accumulations), leptin, high-sensitivity C-reactive protein (hs-CRP) and PAI-1 were higher in the overweight group. In addition, in all groups, these parameters were positively associated with oxidative stress as measured by urinary excretion of 8-epi-PGF2 α . Using an animal model of obesity, Vincent et al. (1999) found that obesity was associated with increased myocardial oxidative stress. In this study, left ventricular tissue from obese Zucker rats had a high concentration of TBARS and hydroperoxide.

The potential mechanism underlying oxidative stress in obesity include hyperglycaemia, increased muscle activity to carry excessive weight, elevated tissue lipid levels, inadequate antioxidant defenses, chronic inflammation, endothelial ROS production and hyperleptinemia (Vincent et al., 2006). These mechanisms have been linked to elevated FFA, blood glucose, cortisol, and angiotensin II, adipocytokines (dysregulation) and increased inflammatory cytokines (TNF- α , IL-6) in obesity (Eriksson et al., 2007). Using adult male Sprague-Dawley rats, Li et al. (2005) have demonstrated that high fat diet-induced obesity is accompanied by increased serum and visceral advanced glycation end products (AGEs), activation of p38 MAPK and apoptosis in the heart and in the liver (Li et al., 2005) which could explain the basic mechanism of obesity-induced tissue damage. In addition, the hexosamine pathway has also been indicated as a possible mediator in hyperglycaemia-induced glucotoxicity (Evans et al., 2005).

A study done by Vincent et al. (2001), has shown that ROS accumulation in the heart was closely associated with myocardial lipid content but not insufficient antioxidant defenses or a greater rate of superoxide production. Therefore, in the high fat diet-induced obesity model, oxidative stress may also include the toxic effects of fatty acid moieties, the generation of toxic by-products of mitochondrial or peroxisomal oxidation, increased oxygen consumption, or fatty acids-induced apoptosis (Finck et al., 2003; Chess & Stanley, 2008).

2.2.2.5.3. Role of oxidative stress in adipose tissue dysfunction and insulin resistance

Increased oxidative stress is associated with adipose tissue dysfunction. This was demonstrated by a study done by Katsuki et al. (2006) where decreased circulating adiponectin levels have been associated with elevated oxidative stress in men. Recently, using 3T3-L1 cells differentiated into adipocytes and then exposed to extraneous H₂O₂, Chen et al.

(2009) have reported that oxidative stress downregulated adiponectin and upregulated PAI-1 and IL-6 expression. Reactive oxygen species (ROS) has been implicated in the aetiology of insulin resistance (Eriksson et al., 2007). Furukawa et al. (2004) suggested that the increased NADPH oxidase and a concomitant reduction in antioxidant enzymes in obesity lead to oxidative stress in remote tissues triggering insulin resistance. At the same time, increased ROS production by adipose tissue leads to oxidative stress in adipose tissue and triggers a consecutive dysregulation of adipocytokines. This dysregulation includes elevated PAI-1, TNF- α , MCP-1 and decreased adiponectin and is associated with insulin resistance (Furukawa et al., 2004).

The increased ROS in the pre-diabetic stage may be due to obesity-induced FFA uptake leading to increased mitochondrial uncoupling and β -oxidation of FFA (Qatanani et al., 2007). In diabetic patients, reactive oxygen species generation is considered as a consequence of insulin resistance-induced hyperglycaemia (Grattagliano et al., 2008). At a molecular level, oxidative stress may lead to insulin resistance by activation of serine /threonine kinases that negatively affect both the secretion of insulin by β -cells and insulin signalling cascades (Evans et al., 2003). The stress-activated kinases include JNK/SAPK, p38 MAPK, PKC α and β , IKK θ , and NF κ B pathways. Activation of these pathways may enhance cytokine production which impairs insulin action (Eriksson et al., 2007).

In view of the above, the question is whether antioxidant supplementation would reverse the detrimental actions of obesity by reducing oxidative stress and leading to subsequent restoration of insulin sensitivity. Accordingly, a study done by Vincent et al. (2009) has demonstrated that antioxidants (combination of vitamin E, vitamin C, and β -carotene) supplementation (8 weeks) moderately lowered the homeostasis model assessment (HOMA) index at 15% in overweight young adults. This was associated with an increase in adiponectin levels and a reduction in plasma lipid hydroperoxide and endothelial adhesion molecules.

2.2.3. Obesity-induced cardiac alterations

Cardiac alterations associated with obesity remain complex (Abel et al., 2008; Harmancey et al., 2008; Mittendorfer & Peterson, 2008; Chess & Stanley, 2008). It has been noticed that metabolic remodelling or metabolic flux alterations may precede functional and structural

alterations (Harmancey et al., 2008). This section will focus on the impact of obesity on cardiac structure, metabolism and function in normoxia and ischaemia/reperfusion injury.

2.2.3.1. Heart environment in obesity

Systemic alterations associated with obesity may affect the heart through various mechanisms. This may include direct effects from adipose tissue secretions and effects from haemodynamic and metabolic factors (Abel et al., 2008; Poirier et al., 2006).

2.2.3.1.1. Humoral or direct factors

Adipose tissue dysfunction in obesity leads to increased oxidative stress and inflammation associated with dysregulation of production of adipose tissue-derived substances (see section 2.2.2.2). Increased oxidative stress results in a compromised heart with respect to its metabolism and function (Abel et al., 2008; Banerjee & Peterson, 2007). Obesity has been described as inflammatory disease with elevated circulating cytokines (Dixon et al., 1990) associated with upregulation of the expression of myocardial pro-inflammatory cytokines after infarction (Thakker et al., 2006).

2.2.3.1.2. Metabolic factors

Obesity-induced metabolic factors include fuel availability and delivery abnormalities. Fuel availability is influenced by insulin resistance, abnormal glucose metabolism (leading to formation of AGEs and glucotoxicity) along with dyslipidaemia (characterized by elevated circulating FFA and a consecutive lipotoxicity) (Chess & Stanley, 2008). Fuel delivery to the obese myocardium is compromised by endothelial dysfunction and coronary artery disease or /and macro vascular disease (Abel et al., 2008; Poirier et al., 2006).

2.2.3.1.3. Haemodynamic factors

It is well established that haemodynamic alterations affect the heart in obesity (Alpert, 2001). These factors affect cardiac structure and function, and include: volume expansion, a subsequent increase in preload and cardiac output and hypertension (Alpert, 2001). Other factors associated with obstructive sleep apnoea syndrome (OSAS) negatively affects left

ventricle filling and can cause ventricular arrhythmias, and eventually heart failure (Kato et al., 2009). OSAS mechanisms include a breathing disorder which induces increased sympathetic activity, renin-angiotensin system activation, and a direct action of metabolic factors on haemodynamic (e.g. apnoea-induced hypoxemia triggers nitric oxide production and a resultant endothelial dysfunction) (Kato et al., 2009). Although haemodynamic mechanisms are important in cardiac alterations associated with obesity (for review see Poirier et al., 2006; Vasan, 2003), we will only focus on cardiac fuel availability and metabolism.

2.2.3.2. Impact of obesity on myocardial metabolism

Obesity and its associated metabolic changes affect myocardial metabolism through complex mechanisms involving adipocytokines signalling, altered insulin signalling, and fuel (glucose and lipid) availability (Lopaschuk et al., 2007). Adipocytokines and insulin signalling have been previously discussed in obesity-induced systemic alterations (section 2.2.1). For reviews on cardiac energy metabolism in the normal heart and in obesity, see Coort et al. (2007), Stanley et al. (2005) and Lopaschuk et al. (2007). Here we give a brief summary focusing on obesity.

The heart is omnivorous and can utilize fatty acids, carbohydrates, and lactate as substrates depending on their concentration in the circulating blood. Cardiomyocytes oxidise predominantly long chain fatty acids (LCFA) (60-70%) while glucose (20%) and lactate (10%) are metabolized to lesser extent. This is due to the substrate selection via the Randle cycle (inhibition of glucose uptake and catabolism by free fatty acid (FFA) oxidation) (Bertrand et al., 2008; Randle et al., 1963). Figure 2.6 represents myocardial metabolism under conditions of elevated fatty acids. Long chain fatty acids enter the sarcolemma by passive diffusion and a protein-mediated transport system by fatty acid transport protein (FATP) or a plasma membrane fatty acid binding protein (FABPm) (Coort et al., 2007; Chess & Stanley, 2008). Once in the cytosol, the non-esterified FFA bind to FABP and are then activated by esterification to fatty acyl-CoA. Long chain fatty acyl-CoA can be esterified into triglycerides or transported across the inner mitochondrial membrane for further β -oxidation.

In obesity, the heart is subjected to increased FFA delivery leading to increased FFA oxidation and reduced glucose utilization (see fig.2.6). Continued excessive FFA uptake overwhelms the FFA accumulation capacity of the heart causing contractile dysfunction (Young et al., 2002). Excess lipid storage contributes to intramyocellular FFA accumulation (steatosis) and

lipotoxicity (via diacyl glycerol/protein kinase C and ceramide pathways), and can cause mitochondrial dysfunction, leading to cell death and consequent left ventricular (LV) dysfunction (Mittendorfer & Peterson, 2008) (see fig.2.6). On the other hand, increased FFA β -oxidation can result in increased reactive oxygen species and contribute to increased oxidative stress, cell death and/or Ca^{2+} handling abnormalities (Dixon et al., 1990), leading to heart failure (Chess & Stanley, 2008). Recently, Lima-Leopoldo et al. (2008) demonstrated that obesity induces upregulation of genes involved in myocardial Ca^{2+} handling. In this study, rats fed with a high fat diet developed metabolic abnormalities including glucose intolerance, hyperinsulinemia and hyperleptinemia, associated with increased gene expression of proteins related to Ca^{2+} transport, SERCA_{2a}, ryanodine receptor 2 (RyR₂) and phospholamban (PLB) without causing changes in sarcolemmal Ca^{2+} handling genes, NCX and Cacna1c (Lima-Leopoldo et al., 2008). Although the mechanism responsible for the alteration in genes responsible for myocardial Ca^{2+} handling is still unknown, it could reflect a compensatory mechanism to the impaired myocardial Ca^{2+} handling evident in obesity (Mittendorfer & Peterson, 2008).

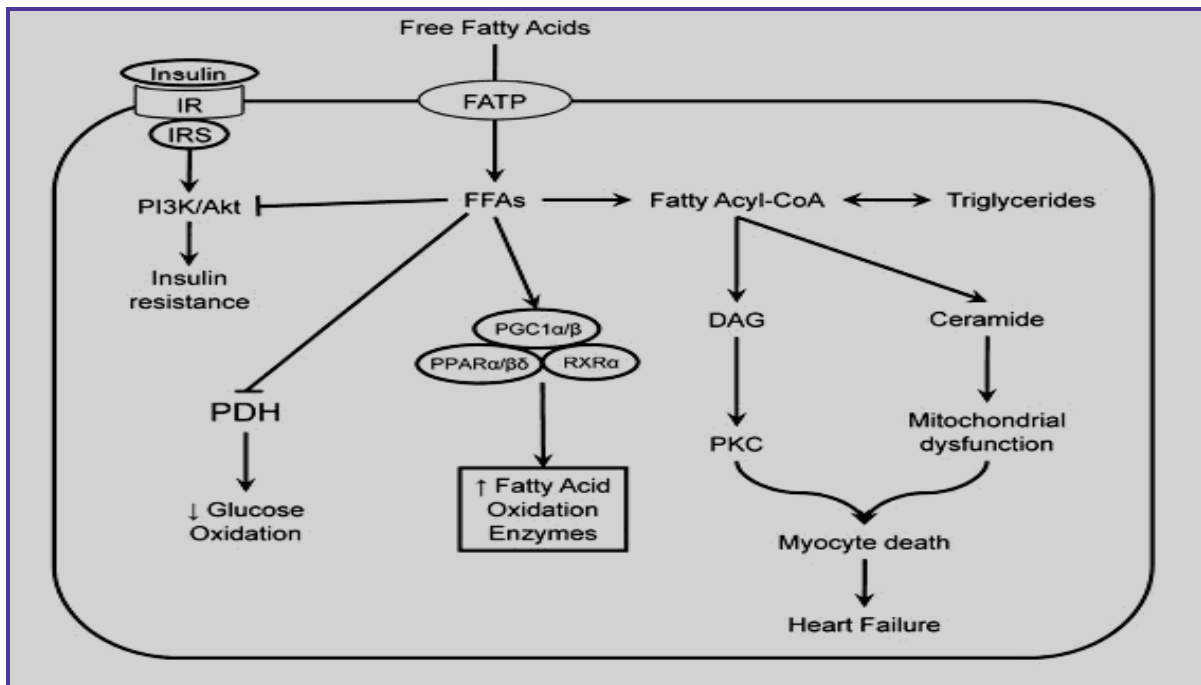


Figure 2- 6 Myocardial metabolism under conditions of elevated fatty acids

(Chess & Stanley, 2008) FATP, fatty acid transport proteins; FFAs, free fatty acids; DAG, diacylglycerol; PKC, protein kinase C; PPAR: Peroxisome proliferator-activated receptor; PDH, pyruvate dehydrogenase; RXR, retinoid X receptor; PGC, peroxisome proliferator-activated receptor- coactivator. IR, insulin receptor; IRS, insulin receptor substrate.

The excess FFA accumulation in the myocardium is also associated with impaired insulin signalling (Chess & Stanley, 2008; Martins et al., 2008) (see fig.2.6). It has been observed that rats fed a high fat diet, compared to their controls, exhibited an impaired myocardial insulin signalling by reducing phosphorylation of protein kinase B (PKB/Akt), decreasing phosphorylation of IRS-1/IRS-2 and associated PI-3K activity with a concomitant hypophosphorylation of phospholamban (PLB) (Ouwens et al., 2005) accompanied by a reduced myocardial acetyl-Co A carboxylase (ACC) phosphorylation (Ouwens et al., 2007).

2.2.3.3. Cardiac hypertrophy/remodelling in obesity

Cardiac hypertrophy refers to an increase in the size of the entire heart, but more commonly, hypertrophy occurs in specific cardiac chambers relative to body size (Abel et al., 2008). Two types of hypertrophy namely concentric and eccentric hypertrophy may occur in response to external stimuli. Concentric hypertrophy indicates an increase in wall thickness relative to chamber size while the eccentric hypertrophy is characterized by chamber enlargement that is more prominent than the increase in wall thickness (Abel et al., 2008).

Cardiac hypertrophy associated with obesity results from a compensatory increase in cardiac output to meet the increased metabolic (oxygen) demand which is associated with excess adipose tissue in obese patients (Poirier et al., 2006). This increased cardiac output is accomplished by increasing stroke volume and as consequence, the left ventricular chamber dilates to accommodate the increased venous return with eventual development of an eccentric or concentric hypertrophy (Vasan, 2003; Mathieu et al., 2008).

While eccentric left ventricular (LV) hypertrophy is commonly present in patients with severe obesity (Poirier et al, 2006), Woodiwiss et al. (2008) recently demonstrated that central obesity was correlated with LV mean wall thickness, concentric LV hypertrophy and remodelling, but not with LV end-diastolic diameter or eccentric LV hypertrophy. From these observations it was concluded that obesity may promote left ventricular concentric rather than eccentric geometric remodelling and hypertrophy independently of blood pressure (Woodiwiss et al., 2008).

Studies in an animal model of diet-induced obesity have shown that compared to their normal controls, hearts isolated from obese rats were hypertrophied as indicated by increased heart weight / body weight ratios (Du Toit et al., 2005) and LV/tibia length (Du Toit et al., 2008).

2.2.3.4. Cardiac function

Obesity is a risk factor for left ventricular diastolic dysfunction, systolic dysfunction, and clinical heart failure and contributes to cardiac dysfunction even in the absence of other frequently encountered cardiovascular risk factors (Poirier et al, 2006). Increased adiposity *per se* independent of atherosclerosis and myocardial infarction impairs both diastolic and systolic function (Wong et al., 2004; Mittendorfer et al., 2008). LV diastolic dysfunction has consistently been shown to be associated with obesity but data on the relationship between LV systolic dysfunction and obesity lack consistency (Abel et al., 2008).

Recently, it has been demonstrated that central obesity measured by body mass index, waist circumference, and waist hip ratio was associated with a lower LV ejection fraction and LV diastolic dysfunction (Ammar et al., 2008). In the study of LV performance in obese patients, Nakajima et al. (1985) demonstrated that alterations in cardiac performance in obese patients with left ventricular enlargement and wall thickening are attributed not only to the excess of body weight but also to the duration of obesity. In this regard, it has been reported that depressed LV function was an early abnormality associated with obesity, and paralleled the degree of obesity (de Divitiis et al., 1981). It is therefore evident that mechanisms leading to cardiac dysfunction include alterations in volume and pressure and myocardial metabolism, which may initially be adaptations that evolve into maladaptive responses over time (Harmancey et al., 2008; Alpert, 2001; Nakajima et al., 1985).

Beside these human studies, convincing evidences using animal models of diet-induced obesity have demonstrated a reduced cardiac performance/efficiency associated with obesity (Akki & Seymour, 2009; Du Toit et al., 2005). Isolated hearts from rats fed with a western diet for 9 weeks showed a decline in myocardial function and a concomitant increase in myocardial oxygen consumption (Akki & Seymour, 2009). Du Toit et al. (2005) have also found that after 16 weeks of feeding rats a high calorie diet, basal function of the hearts from obese rats was significantly depressed compared to age-matched controls.

2.2.3.5. Myocardial ischaemia-reperfusion injury in obesity

Myocardial ischaemia and reperfusion damage has been described in section 2.1. It is characterized by a left ventricular dysfunction, myocardial infarction and arrhythmias. Obesity and related metabolic abnormalities including insulin resistance, dyslipidaemia, and hypertension have been associated with an increased incidence of myocardial infarction (Fedorowski et al., 2009; Prasad et al., 2009; Ranjith et al., 2007). In a study done by Zeller et al. (2008), 70% of people with acute myocardial infarction (AMI) were overweight or obese. In a cross sectional study of 40 men with AMI, using the concentration of total creatine phosphokinase (CPK) and CPK-MB isoenzyme as an estimate of myocardial infarction, Iglesia Bolanos et al. (2009) have reported a strong association between abdominal obesity and myocardial infarction. The same association has been reported by Prasad et al. (2009) showing that among patients with acute myocardial infarction the prevalence of metabolic syndrome was 45%, obesity being the essential feature.

While an *in vivo* animal study by Thim et al. (2006) has reported that the size of myocardial infarction induced by ischaemia/reperfusion is unaltered in rats with metabolic syndrome, an *in vitro* study by Du Toit et al. (2008) found that isolated hearts from obese rats fed a high calorie diet are more vulnerable to myocardial ischaemia/reperfusion injury than control rats fed a standard rat chow for 16 weeks. In the latter study hearts were perfused with glucose only. This increased susceptibility to myocardial ischaemia/reperfusion injury has also been observed in a mouse model of ischaemic cardiomyopathy induced by brief repetitive ischaemic episodes (Thakker et al., 2008): diet-induced obese (DIO) mice showed increased susceptibility to ischaemia and reperfusion injury compared to lean mice. This was due to the development of microinfarctions associated with an exaggerated inflammatory response in hearts from DIO animals. In other studies, the exacerbation of ischaemia and reperfusion injury seen in DIO models compared to their control has also been linked to increased inflammatory markers including chemokines and cytokines (Thakker et al., 2006), elevated serum angiotensin II (Du Toit et al., 2005) and high circulating free fatty acids (FFA) (Thakker et al., 2008). This exacerbation of I/R injury in obesity is also the overall result of a cascade of maladaptive metabolic changes of FFA metabolism (Lopaschuk et al., 2007).

2.2.3.6. Diet-induced obesity models and cardioprotection

Although the diabetic heart in humans is sensitive to ischaemic injury (Fedorowski et al., 2009; Prasad et al., 2009; Ranjith et al., 2007; Zeller et al., 2008), there is a controversy regarding the impact of obesity on ischaemia/reperfusion injury in animal studies. For example, one has to note that contrary to pre-diabetic DIO animals (Du Toit et al., 2008; Li et al., 2008), and type 2 diabetic BBZ rats hearts (Li et al., 2008), hearts from obese diabetic animals displayed a reduced infarct size compared to their non-diabetic control (Bouhidel et al., 2008; Kristiansen et al., 2004; Wang et al., 2004). In addition, comparing lean and obese diabetic rats, although there was no significant difference between infarct sizes of hearts from diabetic rats, Kristiansen et al. (2004) have noted that the post ischaemic haemodynamic recovery was significantly smaller in obese diabetic rats (Zucker diabetic fatty) than in the lean diabetic animals. Furthermore, it was observed that both diabetic rat hearts have a smaller infarct size than the non diabetic control rats (Kristiansen et al., 2004), suggesting a possible protective effect.

To explain these findings, it has been mentioned that the older type II diabetic rats had an impaired cardiac function compared to their age-matched non diabetic rats (Wang et al., 2004). This should explain the different features between these studies: Du Toit et al., 2008 (>16 weeks of feeding) and (Bouhidel et al., 2008; Kristiansen et al., 2004) (< 16 weeks of feeding). Other possible explanations would be the difference in the severity of the ischaemic insult and the substrate presentation. However, this cannot be considered since these studies have been conducted under the same experimental conditions for both groups. The last consideration refers to hyperglycaemia as indicated by Wang et al. (2004), supporting the fact that in *ex vivo* models, the onset of type II diabetes does not increase the sensitivity to ischaemia/reperfusion injury. Therefore, it appears that the observed increased susceptibility to ischaemia/reperfusion injury could be explained by the fact that the glucose levels of obese rat model used by Du Toit et al. (2008) were not significant different from those of control lean rats. In addition, rats were older (Du Toit et al., 2008) than those of other studies (Bouhidel et al., 2008; Kristiansen et al., 2004). The impact of age may explain the increased myocardial susceptibility to I/R injury seen in DIO rats (Du Toit et al., 2008; Li et al., 2008).

Beside this increased myocardial susceptibility to ischaemia/reperfusion injury seen in animal models of diet-induced obesity, studies have demonstrated that cardioprotective strategies effective in normal conditions are impaired in experimental obesity (Balakumar et al., 2009;

Katakam et al., 2007). Using obese Zucker diabetic fatty rats subjected to a preconditioning protocol, Kristiansen et al. (2004) found that post-ischaemic haemodynamic recovery was impaired. Also postconditioning proved to be less effective in obesity. Bouhidel et al. (2008) using obese *ob/ob* mice have demonstrated that hearts from obese animals subjected to post-conditioning protocol exhibited impaired protective responses compared to corresponding controls. In this study, hearts from obese mice have showed an increased infarct size with a concomitant reduction in activation of PKB/Akt and ERK42/44 and AMPK during reperfusion. This has been linked to the loss of cardioprotective effects of leptin in obese *ob/ob* mice (Dixon et al., 2009; Smith et al., 2006) and a strong stimulus has been suggested for an effective post or preconditioning in these animal models (Bertrand et al., 2008; Tsang et al., 2005).

2.3. EFFECTS OF MELATONIN ON THE HEART

2.3.1. Overview

Melatonin or N-acetyl-5-methoxytryptamine is the hormone secreted mainly by the pineal gland, located in the center of the brain, under the control of the central nervous system via the suprachiasmatic nucleus (SCN) of the hypothalamus. The pineal gland is only active in darkness while light suppresses its activity (Nowak & Zawilska, 1998). After secretion, melatonin is released into the circulation and gains access to various fluids, tissues and cellular compartments (Claustrat et al., 2005). As a consequence, the levels of melatonin in the pineal gland and in blood are high at night and low during the day (Altun et al., 2002).

Melatonin was firstly purified and characterized from the bovine pineal gland extract by Lerner et al. (1958). It is a highly conserved molecule found in almost all groups of organisms, from bacteria, plants, protozoa to humans (Hardeland & Fuhrberg, 1996). The molecular structure of melatonin is presented in fig.2.7. In pinealocytes, melatonin is synthesised from serotonin by two enzymes arylalkylamine-N-acetyltransferase and hydroxyindole-O-methyl transferase (AA-NAT). From the blood stream, its precursor amino acid tryptophan is first converted by tryptophan hydroxylase to 5-hydroxytryptophan, which is then decarboxylated to serotonin. The biosynthesis and metabolism of melatonin has recently been reviewed by Pandi-Perumal et al. (2006), Tan et al. (2007), Hardeland (2008) and Zawilska et al. (2009).

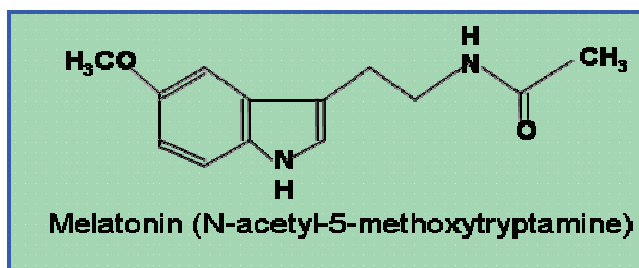


Figure 2- 7 Molecular structure of melatonin

Melatonin is involved in a wide range of physiological functions in humans and animals (for review Pandi-Perumal et al., 2006). It plays the classical role of a chronobiotic or endogenous synchroniser which exhibits a circadian variation controlled by the suprachiasmatic nucleus (SCN), the circadian pacemaker. Melatonin has also been shown to have anti-excitatory, antioxidant, anti-inflammatory, immunomodulatory and vasomotor effects (Hardeland et al.,

2006). Some of these actions are partially subject to circadian variations and an additional interdependence and independence exist between some of the other fields of actions (see fig.2.8). Many of these biological actions of melatonin are produced through activation of membrane receptors (Pandi-Perumal et al., 2006). Because melatonin is a highly lipophilic and hydrophilic compound, it is able to cross all morphological barriers and to act not only in every cell but also within every subcellular compartment. Therefore, some of its intracellular actions are independent of any receptor (for example, free radical scavenging) while others are mediated by nuclear receptors (Carlberg, 2000; Wiesenberg et al., 1998).

It has been demonstrated that the heart is connected to the SCN *via* multisynaptic autonomic neurons, and hence endogenous melatonin may affect heart physiology (Scheer et al., 2001). Melatonin through its anti-adrenergic effects influences blood pressure (Kitajima et al., 2001), myocardial contractility (Abete et al., 1997) and antioxidant reserve (Girouard et al., 2004). In addition, melatonin receptors have been identified in the heart and arteries (Ekmekcioglu et al., 2003; Pang et al., 2002) and decreased melatonin levels were observed in cardiovascular pathological conditions including ischaemic heart disease or myocardial infarction (Dominguez-Rodriguez et al., 2002 & 2005; Yaprat et al., 2003). This section will only focus on the effects of melatonin on the heart, especially its antioxidant properties in experimental ischaemia and reperfusion injury models. The possible underlying mechanisms of its actions will be discussed.

2.3.2. Antioxidant actions of melatonin

Oxidative stress has been shown to play a crucial role in cardiovascular diseases (Dhalla et al., 2000a; Lefer & Granger, 2000; Molavi & Mehta, 2004; Singal et al., 1998). As previously mentioned (section 2.2.2.5.), oxidative stress is characterized by amongst others, DNA damage, lipid peroxidation and oxidized protein (Valko et al., 2007). Alterations to the cellular and subcellular organelles may result in cardiac defects and a subsequent dysfunction, arrhythmias, and infarction, a hallmark of myocardial ischaemia/reperfusion injury (see fig.2.9 p.38) (Dhalla et al., 2000b; Opie, 2004).

Melatonin is able to neutralize a number of toxic reactants including reactive oxygen species and free radicals (Reiter et al., 2000; Reiter et al., 2008). It has also been shown to stimulate antioxidative enzymes (Rodriguez et al., 2004; Vural et al., 2001) and to increase the efficacy

of the classic antioxidants vitamin E, vitamin C and glutathione (GSH) when used in combination with melatonin (Gitto et al., 2001). Furthermore, it has been observed that several metabolites which are formed when melatonin neutralizes damaging reactants, are themselves scavengers (Tan et al., 2001; Tan et al., 2007; Reiter et al., 2007), suggesting that a cascade of reactions increases the efficacy of melatonin when used in pathological conditions associated with increased oxidative stress (Korkmaz et al., 2008; Reiter et al., 2003).

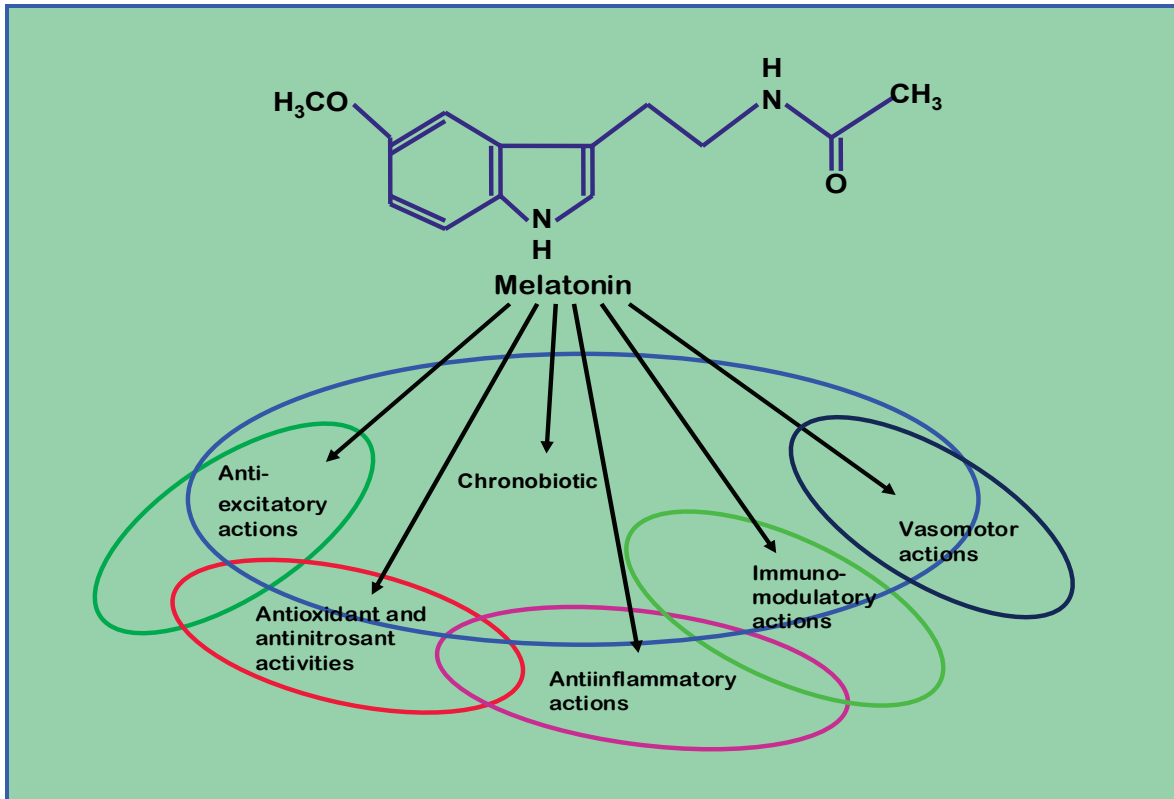


Figure 2- 8 Major fields of actions of melatonin

The chronobiotic actions overlap with all other effects of melatonin and an additional interdependence and independence exist between some of the other areas of actions (Hardeland & Poeggeler, 2008).

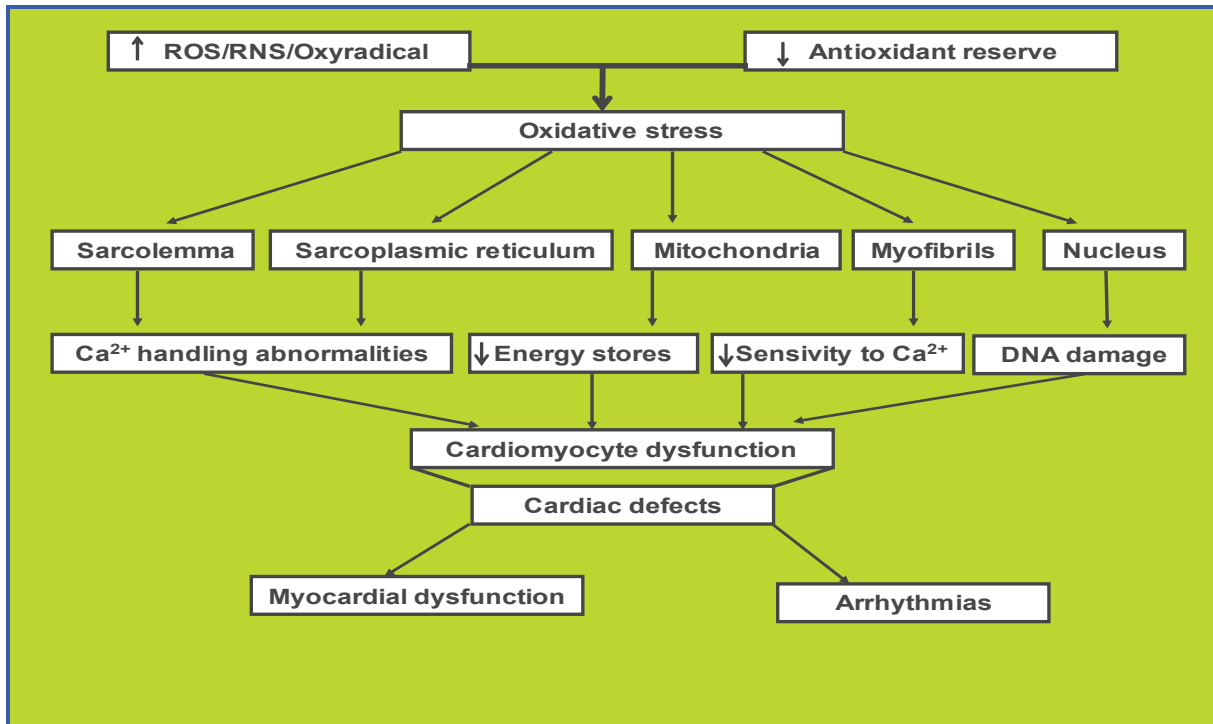


Figure 2- 9 Oxidative stress and cardiomyocyte

A schematic representation depicting the involvement of defects in subcellular organelles due to oxidative stress as a consequence of either increased formation of reactive oxygen species and/or decreased antioxidant reserve (Dhalla et al., 2000a).

2.3.2.1. Free radical scavenging actions (see table 2.1 and fig. 2.11)

Melatonin lowers oxidative cellular and molecular damage through neutralization of free radicals (for review Reiter et al., 2004 & 2009) without being pro-oxidant (Chan & Tang, 1996). The free radical scavenging actions of melatonin were first theorized by Chen et al. (1993) and demonstrated by Tan et al. (1993) in a study showing that melatonin scavenges the hydroxyl radical ($\cdot\text{OH}$). This discovery was subsequently confirmed in cell free systems and *in vivo* (for review Reiter et al., 2000). Indeed, melatonin possesses an electron rich aromatic indole ring and functions as an electron donor thereby reducing and repairing electrophilic radicals (Martinez et al., 2005). Beside the hydroxyl radical, melatonin has been shown to scavenge other free radicals and reactive oxygen species. These include: hydrogen peroxide (Tan et al., 2002), superoxide (Ximenes et al., 2005), singlet oxygen (Matuszak et al., 2003), hypochlorous acid (Zavodnik et al., 2004), peroxy, alkoyl radicals, nitric oxide and peroxyxynitrite anion (Allegra et al., 2003; Hardeland, 2005).

The mechanism by which melatonin interacts with free radicals is only partially understood. The product of the reaction of melatonin scavenging the hydroxyl radical is the indolyl radical which also neutralizes $\cdot\text{OH}$ to produce cyclic 3- hydroxymelatonin (C3-OHM). Once C3-OHM is formed *in vivo*, it is excreted in the urine where it is used as a biomarker of $\cdot\text{OH}$ generation. It has also been shown that C3-OHM can further be oxidized to N¹-acetyl-N²-formyl-S-methoxykynuramine (AFMK). This compound is also able to donate its electrons and neutralize radical species (Tan et al., 2001). AFMK can interact with ROS and RNS to form AMK. AMK can further react with peroxynitrite (ONOO⁻) to form 3-acetamidomethyl-6-methoxycinnolinone (AMMC) and N¹-acetyl-5-methoxy-3-nitro kynuramine (AMNK). Recently, AMK was shown to scavenge singlet O₂ (Schaefer & Hardeland, 2009). Other melatonin metabolites (2- and 6-hydroxymelatonin, 1-nitromelatonin, and nitrosomelatonin) have also been mentioned for their antioxidant effects (Tan et al., 2007). Thus, in view of the cascade of reactions that include AFMK, as pointed out by Tan et al. (2007), a single melatonin molecule can scavenge 10 ROS/RNS molecules. In addition, melatonin and its metabolites have been shown to also reduce electron leakage and free radical formation by the mitochondria (Acuna-Castroviejo et al., 2007; Rodriguez et al., 2007). Importantly, melatonin has been shown to protect the heart by inhibition of the mitochondrial permeability transition pore (MPTP) (Petrosillo et al., 2009).

2.3.2.2. Antioxidant stimulation (see table 2.1 and fig.2.11)

Beside its free radical scavenging properties, melatonin regulates antioxidant activities and gene expression (Rodriguez et al., 2004; Tomas-Zapico & Coto-Montes, 2005). It stimulates the antioxidant enzymes, such as superoxide dismutases (SOD), both MnSOD and CuZnSOD, catalase (CAT), glutathione peroxidase (GPx) (Barlow-Walden et al.,1995), glutathione reductase (GRd) as well as the enzyme glucose-6-phosphate dehydrogenase (G6PD) (Reiter et al., 2000). In addition, melatonin administration down-regulates the pro-oxidant enzymes, such as nitric oxide synthases, lipoxygenases (Hardeland, 2005), myeloperoxidase (MPO) and its downstream inflammatory pathways (Galijasevic et al., 2008). Furthermore, it enhances the levels of intracellular glutathione (GSH) (important antioxidant) by stimulating the rate-limiting enzyme in its synthesis, γ -glutamylcysteine synthase (Urata et al., 1999). GSH is used by Gpx as a substrate to metabolize H₂O₂; in this process GSH is converted to oxidized glutathione (GSSG). To maintain the high levels of GSH, melatonin promotes the activity of glutathione reductase (GRd), which converts GSSG back to GSH (Reiter et al., 2000). NADPH is a cofactor of G6PD in the conversion of GSSH to GSH (Reiter et al., 2007) (see fig.2.11).

It has been observed that serum melatonin levels correlate positively with the enzymatic antioxidant defense system levels (Gumral et al., 2009). Melatonin has been shown to normalize the antioxidant content when lowered by a pathological condition or drug metabolism associated with increased free radical generation in the heart (Cruz et al., 2009; Baydas et al., 2002; Oz et al., 2006). Garcia et al. (2009) have recently reported that melatonin increased the mRNA levels of MnSOD, CAT, and GRd in the presence of the pro-oxidant agent aluminium, independently of the animal model used. These stimulatory effects have been linked to receptor-mediated pathways.

2.3.2.3. Role of melatonin receptors

Beside the non specific direct antioxidant actions of melatonin, melatonin also acts through specific receptors mediated pathways to confer other effects including the regulation and stimulation of antioxidative enzymes (Rodriguez et al., 2004; Tomas-Zapino & Coto-Montes, 2005). In mammals, two types of melatonin receptors, the MT1 and MT2, have been identified in the central nervous system (CNS) and peripheral organs including the heart and the blood vessels (Ekmekcioglu et al., 2003; Pang et al., 2002). These receptors belong to the G-protein-coupled receptor (GPCR) superfamily and are involved in the cardioprotective effects of melatonin (Rezzani et al., 2006; Lochner et al., 2006; Sallinen et al., 2007; Sallinen et al., 2008). A third melatonin-binding site has been purified and characterized as the enzyme quinone reductase 2 (QR2) (Boutin, 2007; Nosjean et al., 2000) but its role in the heart has not yet been identified. In addition, to confer its intracellular actions melatonin may also bind to calmodulin (Soto-Vega et al., 2004), to calreticulin (Hardeland, 2009), and the mitochondrial binding sites (Acuna-Castroviejo et al., 2007) as well as to nuclear receptors of the retinoic acid receptor family including ROR α 1, ROR α 2 and RZR β (Carlberg, 2000; Wiesenberg et al., 1998).

Despite identification of melatonin receptors MT1 and MT2, the signalling pathways in the heart are still poorly understood. It is generally accepted that melatonin activation of MT1/2 receptors, *via* G inhibitory protein, stimulates the phospholipase C pathway leading to increases in cytosolic Ca²⁺. This could in turn lead to the phosphorylation of PKC which activates the transcription factor cAMP responsive element binding protein and activating transcription factor (CREB-ATF), which then modulates immediate early gene (IEG) transcription and gene transcription regulation, and consequently antioxidant enzyme levels

(Rodriguez et al., 2004; Tengattini et al., 2008). This pathway has been implicated in regulation and stimulation of antioxidant enzyme by melatonin (Rezzani et al., 2006).

Melatonin has potent anti-adrenergic effects which could also contribute to its cardioprotective actions. Genade et al. (2008) showed that melatonin significantly reduced both isoproterenol and forskolin- induced cAMP production. Nitric oxide, guanylyl cyclase as well as PKC are involved in the anti-adrenergic effects. Using the appropriate inhibitors (L-NAME, bisindolylmaleimide (BIM) and ODQ, for NO, PKC and cGMP, respectively), these researchers showed that NO and guanylyl cyclase but not PKC are involved in melatonin-induced cardioprotection (see fig.2.10). The study done by Arvola et al. (2006) has also demonstrated the anti-adrenergic effects of melatonin in guinea pig heart papillary muscle.

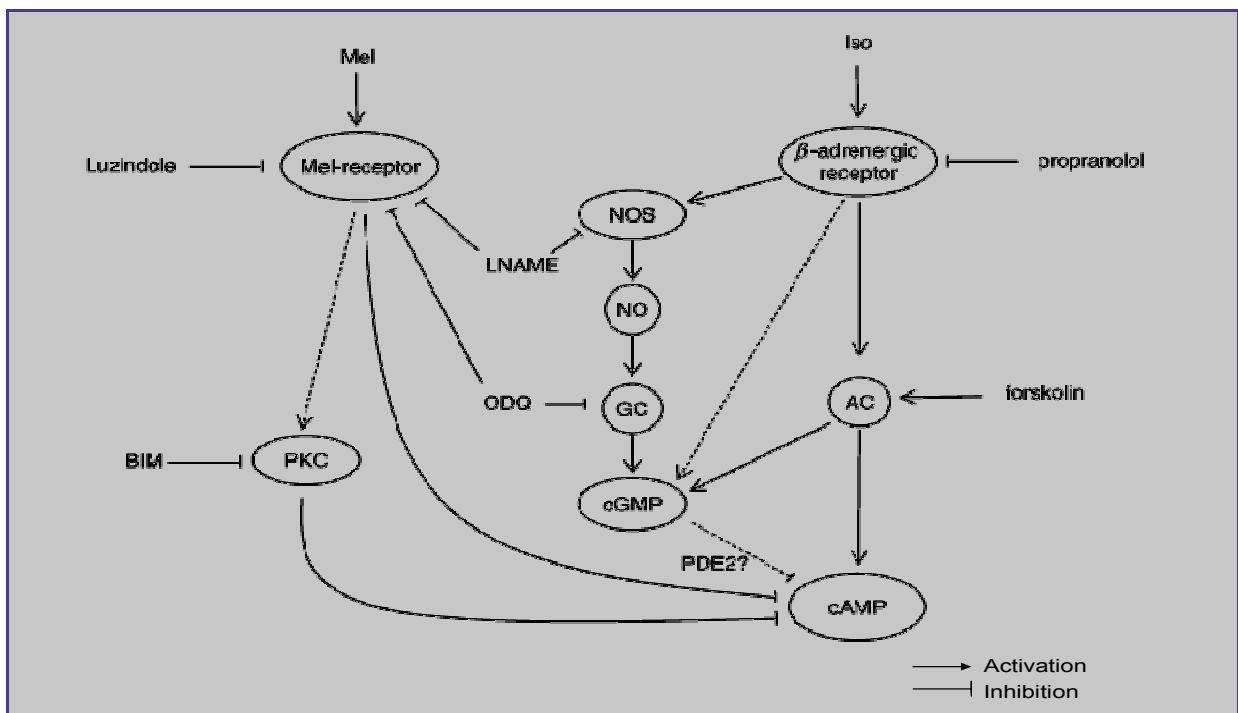


Figure 2-10 Schematic representation of anti-adrenergic effects of melatonin in the isolated rat heart.

Mel, melatonin; Iso, isopreterenol; AC,adenyl cyclase; GC,guanylyl cyclase,PKC, protein kinase C; BIM,bisindolylmaleimide; cAMP,cyclic AMP; cGMP,cyclic GMP; PDE2, phosphodiesterase 2 (Genade et al., 2008).

Table 2- 1 Antioxidant effects of melatonin

Type of actions	Target/site of action	Effects	
Scavenging	ROS	Hydrogen peroxide	↓
		Hydroxyl radical	↓
		Singlet oxygen	↓
		Superoxide anion	↓
	RNS	Nitric oxide	↓
		Peroxynitrite anion	↓
Stimulation	Antioxidant enzymes	Superoxide dismutase	↑
		Catalase	↑
		Glutathione peroxidase	↑
		Glutathione reductase	↑
		Glucose-6-phosphatase dehydrogenase	↑
		Gamma-glutamylcysteine synthase	↑
Inhibition	Pro-oxidant enzyme	Nitric oxide synthase	↓
		Lipo-oxygenase	↓
		Myeloperoxidase	↓

ROS: reactive oxygen species, RNS: reactive nitrogen species. ↑: increase ↓: decrease (Tengattini et al., 2008; Reiter et al., 2003).

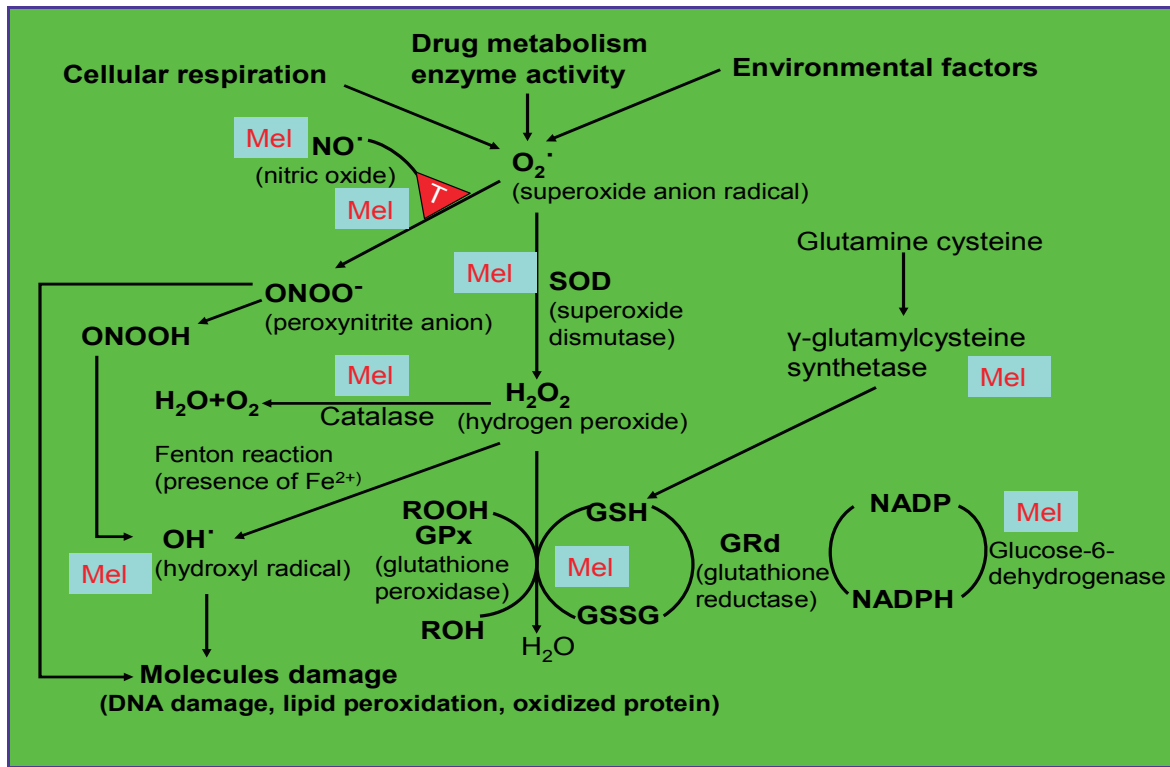


Figure 2- 11 Oxidative stress and sites of action of melatonin (Mel)

Fig.2.11. Melatonin reduces oxidative damage by multiple means. NADP: nicotinamide adenine dinucleotide phosphate; NADPH: the reduced form of NADP; GSH: reduced glutathione; GSSG: glutathione disulfide; ROH: lipid alkoxide; ROOH: organic hydroperoxide; (Kucukakin et al., 2009; Reiter et al., 2003; Korkmaz et al., 2009).

2.3.3. Melatonin and myocardial ischaemia-reperfusion injury

Myocardial ischaemia /reperfusion injury is described in section 2.1. Several *in vitro* and *in vivo* studies have demonstrated the cardioprotective effects of melatonin (for review see Reiter & Tan, 2003; Tengattini et al., 2008). The current literature on the effects of melatonin on myocardial I/R injury is summarized in table 2-2.

2.3.3.1. *In vitro* studies

A study done by Salie et al. (2001) analyzing the effects of melatonin in the setting of hypoxia and re-oxygenation in rat cardiomyocytes, has demonstrated that melatonin protects against myocardial ischaemia/ reperfusion damage *via* inhibition of ROS generation. In this study adult rat ventricular myocytes were preloaded with tetramethylrhodamine (TMRM) in combination with either dichlorodihydro-fluorescein diacetate (DCDHF), dihydrorhodamine 123 (DHR) or fluo 3 to assess the specific actions of melatonin on H₂O₂, ROS and Ca²⁺ using confocal microscopy. Chemical hypoxia, induced by addition of 1.5 mM KCN and 20 mM deoxyglucose to the superfusion buffer resulted in increased H₂O₂, ROS and Ca²⁺ fluorescence. The addition of melatonin (50 to 100 µM) significantly reduced hypoxia-induced morphological damage and H₂O₂, ROS and intracellular Ca²⁺ levels. The reduction of Ca²⁺ by melatonin has also been reported by Chen *et al.* (1993) comparing cardiac tissue incubated with or without melatonin at different doses. The melatonin induced reduction in Ca²⁺ may play an important role in the protective effects against I/R ventricular arrhythmias as observed later by Tan et al. (1998), Lagneux et al. (2000), Kaneko et al.(2000), Szarszoi et al. (2001) and Vazan et al. (2005) using the isolated Langendorff perfused rat heart. Melatonin at concentration of 1, 10 or 50 µM infused during coronary artery occlusion or reperfusion, significantly reduced premature ventricular contractions and/or ventricular fibrillation (Tan et al., 1998). Indeed, when compared to another antioxidant vitamin C (500 µM) (concentration 10 times that of melatonin), melatonin was more effective in preventing cardiac arrhythmias (Tan et al., 1998). In addition, melatonin pre-treatment (10 mg/kg intraperitoneally (i.p))

caused a significant reduction in infarct size, expressed as the percentage of the risk zone (Lagneux et al., 2000). Furthermore, compared to the untreated rat hearts, pre-ischaemic melatonin administration (100 μ M) improved recovery of left ventricular developed pressure (LVDevP) after 30 minutes normothermic global ischaemia (Kaneko et al., 2000). This was accompanied by a significant reduction in ROS generation and lipid peroxide concentration in the heart (Kaneko et al., 2000).

Using the isolated working rat heart preparation, Lochner et al. (2006) showed that melatonin when administered either before or during reperfusion only, significantly improved cardiac output and work performance and reduced infarct size compared to untreated controls. In addition, long-term administration of melatonin (intraperitoneal injection (2.5 or 5.0 mg/kg) or oral administration in drinking water (40 μ g/ml for 7 days) also caused a significant reduction in infarct size of hearts subjected to regional ischaemia. Interestingly, this protection lasted 2 to 4 days after discontinuation of treatment suggesting the long-term therapeutic potential of melatonin. In this study, the protective actions could be abolished by the melatonin receptor blocker, luzindole, suggesting a role for the melatonin receptor in the cardioprotective actions of melatonin.

2.3.3.2. *In vivo* studies

The cardioprotective effects of melatonin have also been demonstrated *in vivo* (Lee et al., 2002; Sahna et al., 2005). Melatonin at both physiological and pharmacological doses was able to reduce the infarct size in the rat (Lee et al., 2002; Sahna et al., 2002a; Sahna et al., 2003; Sahna et al., 2005) and in mouse (Chen et al., 2003) hearts without significantly affecting the haemodynamic parameters such as mean arterial pressure (MAP), heart rate (HR) and blood pressure (BP) (Sahna et al., 2002a; Sahna et al., 2005). Lee et al. (2002) showed that pre-treatment of rats with intermediate or high doses of melatonin (intravenous bolus of 1.0 and 5.0 mg/kg) 10 minutes before left coronary artery occlusion, significantly suppressed ventricular tachycardia (VT) and fibrillation (VF), and improved the survival rate of rats when compared with untreated controls. These melatonin effects were accompanied by a significant reduction in superoxide anion production and myeloperoxidase (MPO) activity (an index of neutrophil infiltration in the ischaemic myocardium). Chen et al. (2003) using the mouse heart reported a reduction in infarct size by the dose range of 75-150 μ g/kg. The high dose of 150 μ g/kg did not confer an additional protective effects and melatonin was more effective between

0.5 to 2 hours after administration but not at 4 to 24 hours as it was recently shown in isolated rat heart (Lochner et al., 2006). In contrast to the above, using 8-methoxy-2-propionamidotetralin, a melatonin receptor agonist, they failed to demonstrate the involvement of melatonin receptors in cardioprotection.

The role of endogenous melatonin in cardioprotection was first demonstrated in the study done by Sahna et al. (2002b) which showed that the incidence of mortality resulting from irreversible ventricular fibrillation (VF) was significantly higher in the pinealectomized rats (63%) than in the control group (25%). Melatonin administration (0.4 mg/kg, either before ischaemia or reperfusion) to pinealectomized rats significantly reduced the incidence of total (irreversible plus reversible) and irreversible VF (Sahna et al., 2002b). In another study, Sahna et al. (2005) have also demonstrated that melatonin (10 mg/kg) given 10 minutes before ischaemia *via* the jugular vein significantly reduced the infarct size. This protection was accompanied by a reduction in MDA and an increase GSH levels. Ceyran et al. (2008) showed that administration of the high dose of melatonin (50 mg/kg) to rats before ischaemia or reperfusion was characterized by a reduction in cardiac troponin, MDA, MPO levels and an increase in SOD levels. In addition, there was a decrease in Fas expression, myofibrillar oedema, vascularity, inflammatory infiltration and an increased Bcl-2 expression. Recently, Chen et al. (2009) using both *in vitro* and *in vivo* models, showed that independently of glutathione peroxidase (GPx) activation melatonin protected against myocardial I/R injury and apoptosis in both deficient (GPx *-/-*) and wild type (Gpx *+/+*) mice as indicated *in vitro* by a significant improvement in left ventricular end-diastolic pressure and a decrease in lactate dehydrogenase (LDH) levels.

2.3.3.3. Myocardial I/R injury in pathological conditions

In cardiomyopathic hamsters, melatonin reduced ventricular arrhythmias and preserved capillary perfusion during I/R injury (Bertuglia & Reiter, 2007). In this study the animals received melatonin (6 mg/kg) daily for 3 weeks before induction of I/R. During I/R oxidative and nitrosative stress, vasoconstriction, leukocyte adhesion, and vascular permeability were reduced while capillary perfusion was increased in melatonin treated group. It was concluded that melatonin prevents both microvascular injury and ventricular arrhythmias during post-ischaemic reperfusion by modulating the lipid peroxide overproduction and nitrosative stress which are involved in the development of cardiomyopathy (Bertuglia & Reiter, 2007).

In chronically hypoxic rats, melatonin conferred cardioprotection by amelioration of Ca^{2+} homeostasis (Yeung et al., 2008). Melatonin treatment significantly reduced infarct size, MDA generation, and LDH release. The cytosolic calcium levels in isolated cardiomyocyte were also reduced by melatonin treatment. In addition, sarcoplasmic reticulum Ca^{2+} -ATPase2 (SERCA2a) expression was significantly reduced in the melatonin treated hypoxic rats group compared to untreated hypoxic rats while the levels of the protein expression of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and ryanodine receptor (RyR2) in chronically hypoxic rats were not different from those of the normoxic controls (Yeung et al, 2008).

The study by Sallinen et al. (2007) has shown in rats that a myocardial infarction (MI) increased the amount of LV MT2 receptor proteins, MT1 and MT2 mRNA levels. In addition, using the same model of MI, long-term post-infarction subcutaneous melatonin (4.5 mg/kg per day) administration significantly reduced the expression of dihydropyridine receptor (DHPR), the ryanodine receptor (RyR(2)), and sarcoplasmic reticulum Ca^{2+} -ATPase2 (SERCA2a) mRNA. This was associated with an increase in amount of MT2 receptor and atrial natriuretic peptide (ANP) in the LV (Sallinen et al., 2008). This study demonstrated that in response to the MI, melatonin synthesis in the pineal gland may increase rapidly and melatonin receptors may be involved in mediating, at least, in part, the protective effects of melatonin in the heart after infarction. Melatonin receptors, particularly MT2, might contribute to the post-infarction cardioprotective actions of melatonin associated with improved myocardial contractility (Sallinen et al., 2007).

In contrast to the substantial evidence for the cardioprotective effects of melatonin, one study in rabbits failed to demonstrate these effects (Dave et al, 1998). In addition, because of its free radical scavenging properties melatonin prevents preconditioning-induced cardioprotection in isolated rat hearts (Genade et al., 2006).

2.3.3.4. Melatonin and mitochondria in myocardial I/R injury

Mitochondria are known to be involved in the process of cell death (necrosis and apoptosis) following reperfusion (Makazan et al., 2007). Mitochondrial respiration, mainly at the level of complex I and III, is an important source of ROS generation and hence a potential contributor to cardiac reperfusion injury (Das, 1994). It has recently been proposed that melatonin also protects against ischaemia/reperfusion-induced oxidative damage to mitochondria in the heart

(Giacomo & Antonio, 2007; Petrosillo et al., 2006; Petrosillo et al., 2009). Petrosillo and coworkers (2006) showed that melatonin reduced the rate of mitochondrial oxygen consumption, complex I and complex III activity, H₂O₂ production. The degree of lipid peroxidation, cardiolipin content, and cardiolipin oxidation was also reduced. In addition, melatonin has recently been shown to protect the isolated heart against I/R injury by inhibition of mitochondrial permeability transition pore opening (Petrosillo et al., 2009). Interestingly, melatonin was able to prevent the impairment of mitochondrial homeostasis and restored ATP production in septic mice hearts (Escames et al., 2007; Rodriguez et al., 2007).

2.3.3.5. Mechanism of cardioprotection

The mechanisms of the cardioprotective effects of melatonin are still under investigation. It has recently been shown that melatonin administered before or during early reperfusion activates the reperfusion injury salvage kinases (RISK) (PKB/Akt and ERK 42/44) pathway during early reperfusion (Genade et al., 2008). This cardioprotection by melatonin was also recently linked to the inhibition of mitochondrial permeability transition pore (MPTP) opening (Petrosillo et al., 2009) and the activation of the signal transducer and activator of transcription-3 (STAT-3) (Lecour S, Unpublished observation).

2.3.4. Melatonin and left ventricular remodelling

Left ventricular (LV) remodelling refers to left ventricular hypertrophy. It is associated with haemodynamic overload and/or increased oxidative stress. Melatonin was shown to reduce blood pressure, oxidative load and to increase nitric oxide bioavailability, hence conferring the anti-remodelling potential (Simko et al., 2009). In this study, the activity of NOS and endothelial NOS expression increased in the left ventricles of hypertensive animals compared with controls. Melatonin was shown to increase NOS expression, reduce NF- κ B expression and decrease conjugated diene concentration. This was associated with a reduction in collagenous protein concentration and hydroxyproline content in the left ventricle without affecting the LV hypertrophy, demonstrating its antifibrotic rather than its antihypertrophic effects (Simko et al, 2009). These protective actions that reverse LV fibrosis, but not LV hypertrophy in spontaneously hypertensive rats may be caused by melatonin's prominent antioxidative effect (Simko et al., 2009; Paulis et al., 2009). Nevertheless, melatonin was able

to prevent cardiac hypertrophy in hyperthyroid rats along with a reduced oxidative load and altered expression of metabolically important genes including glucose transporter (GLUT-4) (Ghosh et al., 2007).

2.3.5. Conclusion

There are over 15073 Pubmed citations related to melatonin; therefore, it is not possible to mention all data which document the effects of melatonin and its possible clinical importance. Melatonin protects the heart in pathological conditions associated with elevated increased oxidative stress. It most likely acts through its direct free radical scavenging activities and its indirect actions in stimulating antioxidant enzymes *via* membrane or intracellular receptors.

2.4. MELATONIN EFFECTS IN OBESITY

2.4.1. Introduction

Advancing age is characterized with elevated oxidative stress and increases in visceral fat accumulation, plasma insulin and leptin levels (Wolden-Hanson et al., 2000; Rasmussen et al., 1999). Endogenous melatonin levels are reduced with ageing (Pang et al., 1990; Reiter, 1992). These age-associated changes are often associated with obesity-related metabolic or physiological alterations including atherogenic dyslipidaemia, raised blood pressure, and a pro-inflammatory state (Eckel et al., 2005). However, the role of melatonin in obesity is not well established. This section will summarize the effects of melatonin on obesity focusing on body weight gain, adiposity, lipid profile, hormones and glucose levels.

2.4.2. Melatonin effects in non pathological conditions

Melatonin is involved in many physiological processes including amongst others energy metabolism (Pandi-Perumal et al., 2006; Bartness et al., 2002). In middle-aged rats daily melatonin supplementation has been shown to increase plasma melatonin and to reduce body weight, visceral adiposity, and plasma insulin and leptin levels to youthful levels (Wolden-Hanson et al., 2000; Rasmussen et al., 1999). In young rats, long-term oral melatonin administration (4 µg/ml in drinking water) also reduced body weight gain, visceral fat weight, without any alteration of food intake (Kassayova et al., 2006; Bojkova et al., 2006). Melatonin effects on the heart weight are sex dependent. Melatonin increased (in females) or decreased (in males) the heart weight (Markova et al., 2003). It also increases (in males: Bojkova et al., 2006) or reduces (in both males and females) (Kassayova et al., 2006) or has no effect on (in males and females: Markova et al., 2003) serum glucose levels.

The action of melatonin on glucose metabolism is not clear. It varies from one animal model to another. It has been demonstrated that pinealectomy increased glycaemia and melatonin administration, after pinealectomy, normalized blood glucose (Rodriguez et al., 1989; Pruet-Marcassus et al., 2003). In contrast to this, the study done by Bizot-Espiard et al. (1998) reported that neither pinealectomy nor melatonin administration in pinealectomized animals had any effects on serum glucose. Further more, surprisingly, an acute administration of melatonin increased serum glucose (Fabis et al., 2002), suggesting a role in carbohydrate

metabolism. However, the exact mode of action of melatonin in regulation of carbohydrate/lipid metabolism is not clear. It appears that the melatonin effects depend on the dose, mode and time of administration as well as the age, sex, and strain of the animal model (Bojkova et al., 2006; Kassayova et al., 2006; Markova et al., 2003; She et al., 2009). A direct as well as an indirect action via the sympathetic nervous system has been suggested to explain the observed effects of the hormone in obesity (Pruet-Marcassus et al., 2003).

2.4.3. Melatonin role in obesity

Melatonin has been considered as an effective treatment for obesity (Barrenetxe et al., 2004). It has been shown that the amplitude of the nocturnal pineal melatonin peak is significantly decreased in overweight and obese rats (Cano et al., 2008). In a rat model of diet-induced obesity with a high-fat diet, daily melatonin administration significantly decreased body weight (Pruet-Marcassus et al., 2003; She et al., 2009). This decrease in body weight was associated with a reduction in plasma glucose, leptin and triglycerides levels, and was observed as soon as five days after the initiation of daily melatonin treatment and continued throughout the entire time-course of melatonin treatment (Pruet-Marcassus et al., 2003).

A study done by Hussein et al. (2007) in a rabbit model of obesity has demonstrated that intake of melatonin was associated with amelioration of both metabolic and morphological pathologies related to obesity. In this study, when compared with the obesity group, intake of melatonin (treated group) was associated with: a significant decrease in blood pressure, heart rate, sympathetic nerve activity, body weight, food consumption, serum lipids, blood glucose levels and atherogenic index. These changes were accompanied with increased levels of glutathione peroxidase (GSH-Px) and high density lipoproteins (HDL). In addition, there was a disappearance of fatty alterations in the liver and kidney as well as atheromatous changes in the blood vessels of the obese treated group.

In addition to melatonin's efficacy in treating diet-induced obesity, melatonin is also effective in treating middle-aged induced-obesity (Wolden-Hanson et al., 2000). For example, daily treatment with melatonin significantly decreased body weight in middle-aged rats (Rasmussen et al., 1999). Importantly, this melatonin-induced body weight decrease in middle-age was due to a significant decrease in fat content as opposed to lean body mass, and was accompanied by an increase in energy expenditure as expressed by elevated physical activity and body core

temperature, similar to that of young rats (Wolden-Hanson et al., 2000), further suggesting the effectiveness of melatonin for the treatment of obesity.

2.4.4. Melatonin and insulin resistance

Melatonin regulates insulin secretion, lipid and glucose metabolism (Nishida et al., 2005). Melatonin and its agonist NEU-P11 increase insulin sensitivity and improve the metabolic profile in high fat/high sucrose-fed rats (She et al., 2009). This was accompanied by a reduction in oxidative stress and a stimulation of the antioxidant status with a decrease in glucose levels (She et al., 2009). Similar effects were also reported in type I (Akskoy et al., 2003; Vural et al., 2001) and II (Nishida et al., 2002) diabetic rats with no effect of glucose levels (Klepac et al., 2005). Pinealectomy increases (after 21 weeks) plasma insulin and accumulation of triglycerides followed later by a net decrease (after 35 weeks) in insulin levels (Nishida et al., 2003). This reflected impairment in insulin release from pancreatic β -cells, as seen in patients at the advanced stage of type 2 diabetic mellitus (Kuzuya et al., 2002). Recently, it has been shown that pineal melatonin synthesis is decreased in type 2 diabetic Goto–Kakizaki rats (Frese et al., 2009). Nishida and co-workers (2002) observed that administration of melatonin to rats with type 2 diabetes mellitus reduces their plasma hyperlipidaemia, hyperinsulinaemia and hyperleptinaemia, and improves the conditions associated with type 2 diabetes. Melatonin has been shown to inhibit insulin secretion by pancreatic β -cells (Peschke et al., 2008). Recently, a case study done by Nieuwenhuis et al. (2009) has confirmed the beneficial effects of melatonin in diabetes by successfully treating a 40-year-old woman with insulin-dependent diabetes mellitus with phototherapy.

2.4.5. Melatonin and leptin

Leptin, an adipocyte hormone, has a central role in regulating food intake, body weight and energy expenditure. Pinealectomy increases serum leptin (Wolden-Hanson et al., 2000). It has been observed that exogenous melatonin decreases serum leptin levels in both pinealectomised and intact rat models of diet-induced obesity (Canoplat et al., 2001; Wolden-Hanson et al., 2000) before decreasing plasma insulin (Pulchalski et al., 2003). In young and middle aged rats melatonin did not shown any effect on serum leptin levels (Bojkova et al., 2008). Melatonin acts directly on adipocytes and enhances the leptin expression in the presence of insulin (Alonso-Vale et al., 2004).

Table 2-2 CURRENT LITERATURE ON THE EFFECTS OF MELATONIN ON MYOCARDIAL ISCHAEMIA/REPERFUSION INJURY

Types of studies	Model	Duration of I/R	Melatonin actions	Reference
<i>In vitro</i>	isolated rat heart (Langendorf model)	10/10 (min/min)	↓ premature ventricular contraction and fibrillation	Tan et al., 1998
<i>In vitro</i>	isolated rat heart (Langendorf model)	30/30 (min/min)	↓ ventricular tachycardia and fibrillation; restored ventricular function; ↓ lipid peroxidation; lowered OH [•] generation	Kaneko et al., 2000
<i>In vitro</i>	isolated rat heart (Langendorf model)	5/30 (min/min)	↓ reperfusion arrhythmias; ↓ infarct size	Lagneux et al., 2000
<i>In vitro</i>	isolated rat heart (Langendorf model)	20/40 (min/min)	↓ fibrillation; low concentration had no effect on I/R of the isolated perfused heart rat	Szarszoi et al., 2001
<i>In vitro</i>	Cardiomyocyte in culture (rats)	12.5 or 27.5/1.5	Reduced morphological damage, lowered oxygen free radical generation, reduced intracellular [Ca ²⁺]	Salie et al., 2001
<i>In vitro</i>	isolated and perfused working rat heart	30/45 (min/min)	Significant improvement of hemodynamic parameters and ↓ post-ischemic arrhythmias during reperfusion; ↓ significantly incidence of apoptotic cells	Dobsak et al., 2003
<i>In vitro</i>	isolated rat heart / working heart	20/30 (min/min)	Global ischemia (GI): protective effects during reperfusion, abolishes cardioprotection before and during ischemia preconditioning protocol	Genade et al., 2006

Types of studies	Model	Duration of I/R	Melatonin actions	Reference
<i>In vitro</i>	Isolated working rat heart	20/30 (GI) or 35/30(RI) (min/min)	Before and during reperfusion: improved cardiac output and work performance; ↓ infarct size; long term significantly ↓ infarct size (RI). Luzindole abolishes these effects. Melatonin receptor is suggested to be involved in melatonin cardioprotection.	Lochner et al., 2006
<i>In vitro</i>	Langendorff apparatus	30(GI)/120 or 15 (min/min)	Increased content of cardiolipin with reduced peroxidized cardiolipin, Reduced rates of mitochondrial oxygen consumption, complex I and complex III activity, and H ₂ O ₂ production; ↑ post-ischaemic LVDp and ↓ LVED p	Petrosillo et al., 2006
<i>In vitro</i>	Langendorff and working heart	35 RI/30 or 120 (min/min)	Anti β-adrenergic effects: reduced IFS, ↓NOS & cAMP; increased PKB/Akt, reduced p38 MAPK activity.	Genade et al., 2008
<i>In vitro</i>	Isolated heart rat (Langendorff method) (chronic hypoxic rats)	30/120 Min/min	infarct size reduction, improve Ca ²⁺ handling by preserving SERCA expression, lowered MDA levels, lactate dehydrogenase (LDH)	Yeung et al.,2008
<i>In vitro</i>	Langendorff apparatus	30 GI /120 or 15 (min/min)	Cardioprotection by inhibition of MPTP opening. Before and during reperfusion: ↓ IFS, improves function recovery (↑ LVDp and ↓ LVED p), ↓necrosis (↓LDH); reduction to MPTP sensitivity by increasing resistance to Ca ²⁺ , prevention of NAD ⁺ release and mitochondrial cytochrome-c release	Petrosillo et al., 2009

Types of studies	Model	Duration of I/R	Melatonin actions	Reference
<i>In vivo & in vitro</i>	In situ mice & isolated mice heart (Langendorff method)	50min/4 hours 40/45 (min/min)	Cardioprotection independently of GPx activation. ↑LVEDp and ↓ LDH level , ↓IFS	Chen et al., 2009
<i>In vivo</i>	in situ rat heart	45/60 (min/min)	↓ ventricular tachycardia and fibrillation; lowered total ventricular contractions, suppression of superoxidase production (\dot{O}_2) and lowered myloperoxide (MPO) activity, lowered infarct size, increased survival	Lee et al., 2002
<i>In vivo</i>	in situ rat heart	7/7 (min/min)	↓ ventricular fibrillation, lowered mortality	Sahna et al., 2002b
<i>In vivo</i>	in situ rat heart	3/120 (min/min)	↓ infarct size	Sahna et al., 2002a
<i>In vivo</i>	in situ rabbit heart	30/180 (min/min)	No improvement in cardiac function, no reduction in infarct size	Dave et al., 1998
<i>In vivo</i>	In situ mice heart	60 /4 (min/hours)	Melatonin treatment 30 min before ischemia, ↓ significantly the infarct size/risk area. Mel treatment after ischemia was not protective.	Chen et al.,2003
<i>In vivo</i>	in situ heart rat	30/120 (RI) (min/min)	Regional ischaemia(RI):↓ infarct size,↓ MDA values, ↑GSH levels	Sahna et al., 2005

Types of studies	Model	Duration of I/R	Melatonin actions	Reference
<i>In vivo</i>	in situ heart rat (chronic nitric oxide synthase inhibited rats)	30/120 (RI) (min/min)	Regional ischaemia (RI): ↓ infarct size & BP	Deniz et al., 2006
<i>In vivo</i>	In situ heart cardiomyopathic hamsters	30/30 (min/min)	↓ post ischaemic VT and VF, lowered % lethality ; ↓ oxidative stress (lipid peroxidation: TBARS) and nitrosative stress (nitrite/nitrate), leukocyte adhesion, vascular permeability, increased capillary perfusion, reduced MAP.	Bertuglia et al., 2007
<i>In vivo</i>	in situ heart rat (chronic nitric oxide synthase inhibited rats)	30/120 (RI) (min/min)	Regional ischaemia (RI): ↓ infarct size & BP; ↓ MDA values, ↑MPO levels	Sahna et al., 2008
<i>In vivo</i>	In situ heart rat	20/20 (min/min)	↓cardiac troponin (cTn-T), MDA, MPO levels ↑ SOD levels, Bcl-2 expression ↓Fas expression, oedema myofibrils, vascularity, inflammatory infiltration	Ceyran et al., 2008

↓ decrease; ↑ increase; *GI*, global ischaemia, *RI*, regional ischaemia; *I/R*, ischaemia reperfusion, *MPTP* mitochondrial permeability transition pore ; *SERCA2a*, sarco-(endo)-plasmic reticulum Ca^{2+} -ATPase2; *LDH*, lactate dehydrogenase; *VT*, ventricular tachycardia; *VF*, ventricular fibrillation ; *MPO*, myeloperoxidase; *SOD*, superoxide dismutase ; *MAP*, mean arterial pressure; *HR*, heart rate; *BP*, blood pressure; *GSH*, reduced glutathione; *LVDp*, left ventricular developed pressure; *LVEDp*, left ventricular end-diastolic pressure; *TBARS*, thiobarbituric acid reaction substance; *MDA*, malondialdehyde.

2.5. MOTIVATION FOR THE STUDY

2.5.1. Problem statement

As previously discussed, obesity is a major risk factor for myocardial infarction, one of the common consequences of ischaemic heart disease. The mechanistic link between obesity and ischaemic heart disease is complex and not yet fully understood. High circulating free fatty acids can induce cellular dysfunction by increasing mitochondrial reactive oxygen species (ROS) generation leading to elevated systemic (Furukawa et al., 2004; Vincent et al., 2006) and myocardial oxidative stress (Vincent et al., 1999; Finck & Kelly, 2002; Chess & Stanley, 2008) in obesity. Oxidative stress (elevated free radicals generation) plays an important role in the pathogenesis of cardiovascular disease in general (Dhalla et al., 2000a), and myocardial ischaemia/reperfusion injury in particular (Opie, 2004; Yellon & Hausenloy, 2007). In addition, it has a crucial role in development of metabolic syndrome which in turn increases the incidence of myocardial infarction in obesity (Anand et al., 2008; Iglesias Bolanos et al., 2009). Diet-induced obesity has been shown to increase myocardial susceptibility to ischaemia/reperfusion (I/R) injury in the rat model (Du Toit et al., 2008), demonstrating the strong association between more severe myocardial infarction (MI) and obesity in humans (Yusuf et al., 2005).

Nowadays, there is a strong recommendation to use the antioxidant melatonin in the management of myocardial infarction (Korkmaz et al., 2009; Reiter et al., 2008). Indeed, melatonin is involved in a wide range of physiological functions in humans and animals including amongst other antioxidant, anti-inflammatory, anti-excitatory, and vasomotor effects (Pandi-Perumal et al., 2006). It has been shown that subjects with myocardial infarction have low circulating melatonin levels (Dominguez-Rodriguez et al., 2002) and pinealectomized rats have been shown to have a bigger post-ischaemic myocardial infarction than non-pinealectomized rats (Sahna et al., 2002a). Acute melatonin treatment protects the heart against I/R injury (Reiter & Tan, 2003; Petrosillo et al., 2009; Lochner et al., 2006; Genade et al., 2008; Tengattini et al., 2008). The long-term administration of melatonin also reduces myocardial infarction (Lochner et al., 2006) in lean animals, indicating its potential long-term therapeutic utility. However, the effects of melatonin still remain far from being completely elucidated. Melatonin not only has direct cardiac benefits but chronic administration of melatonin was associated with amelioration of physiological changes related to obesity such

as decreases in body weight gain, blood pressure, circulating glucose, insulin, leptin and lipid levels as well as increase in antioxidant enzymes (Hussein et al., 2007; Prunet-Marcassus et al., 2003; She et al., 2009), suggesting a possible role for melatonin in obesity management. However, to our knowledge, the effects of melatonin treatment on the heart in obesity remain unknown. In addition, the preventive effects of chronic melatonin before the establishment of obesity in animal model of diet-induced obesity have not been studied. Furthermore, the signalling mechanisms of the cardioprotective effects of melatonin have not yet been completely established.

The aim of this study was therefore to investigate the effects of chronic melatonin treatment on myocardial function and susceptibility to ischaemia-reperfusion injury in obesity. We also wished to elucidate some of the potential mechanisms responsible for these cardiac effects.

2.5.2. Hypothesis

We hypothesized that a high calorie diet will induce obesity which, would increase myocardial susceptibility to ischaemia/reperfusion injury (I/R) and exacerbate post ischaemic myocardial infarction and, by virtue of its antioxidant effects, chronic melatonin treatment before the establishment of obesity will prevent the development of insulin resistance and attenuate the susceptibility to I/R injury as well as the post-ischaemic myocardial infarction.

2.5.3. Specific aims

The specific aims of this study were to:

1. Investigate the effects of chronic melatonin administration on the development of diet-induced systemic metabolic alterations. Physiological parameters measured were body and heart weight, circulating glucose, lipids, insulin, and leptin levels. Oxidative stress was assessed by measuring serum lipid peroxidation and glutathione (GSH) levels.
2. Determine whether chronic melatonin treatment protects the myocardium against ischaemia/ reperfusion injury. End-points investigated were infarct size and function recovery.
3. Determine whether melatonin treatment confers cardioprotection by altering the reperfusion injury salvage kinase (RISK) (i.e. PKB/Akt and ERK p42/p44), p38 MAPK and AMPK as well as GLUT-4.

CHAPTER THREE

MATERIALS AND METHODS

3.1. ANIMALS

Age and weight matched male Wistar rats were used in this study. All animals were obtained from the University of Stellenbosch Central Research Facility. They received free access to water and food under the 12-h dark/light cycle (light from 6:00 a.m. to 6:00 p.m.) with temperature and humidity kept constant at 22°C and 40%, respectively. This study was assessed and approved by the Committee for Ethical Animal Research of the Faculty of Health Sciences, University of Stellenbosch, and animals were treated according to the “Guide for the Care and Use of Laboratory Animals”, published by US National Institutes of Health (NIH publication no 85-23, revised 1985).

3.2. STUDY DESIGN

This study was divided in 3 major phases (see fig.3.1). The first, before any intervention, consisted of grouping, feeding and treatment. During the second phase experimental procedures including anthropometric measurements, perfusion of the hearts as well as blood and heart tissue collection were performed. The third phase included biochemical analyses of collected samples (see fig.3.1).

3.2.1. Grouping, feeding and treatment

Male rats weighing 200±20 g were randomly allocated to the following groups: group C, control rats receiving a standard commercial rat chow and drinking water without melatonin; group CM, control rats receiving a standard commercial rat chow and melatonin in drinking water; group D, diet-induced obesity (DIO) rats, receiving a high calorie diet and drinking water without melatonin; group DM, diet-induced obesity rats, receiving a high calorie diet and melatonin in drinking water. Animals were fed and treated for a period of 16 weeks (see fig.3.1). Food and drinking water with or without ethanol or melatonin was replaced daily to avoid fermentation of wet food.

Obesity was induced as previously described (Du Toit et al., 2008). Diet-induced obesity groups (D and DM) were fed a high-calorie diet consisting of 65% carbohydrates, 19% protein and 16% fat. Control groups (C and CM) were fed a standard rat chow (SRC) consisting of 60% carbohydrate, 30% protein and 10% fat. The high calorie diet was prepared containing 33% SRC and 33 % sweetened full cream condensed milk (Clover[®]), 7% sucrose and 27% water. It has been previously shown in our laboratory that rats receiving a SRC and a high calorie diet consumed 371 ± 18 and 570 ± 23 kJ of energy per day, respectively (Du Toit et al., 2008).

3.2.2. Melatonin administration

Melatonin (Sigma Aldrich, St Louis, MO, USA) was orally administered (Lochner et al., 2006). It was first dissolved in small amount of absolute ethanol and then added to drinking water at final concentration of 0.05% (v/v) ethanol with melatonin (60 $\mu\text{g/ml}$ or 40 $\mu\text{g/ml}$). The drinking water intake of control rats was higher than that of obese rats probably due to the fact that the diet was essentially a liquid. In order to achieve a final dosage of 4 mg/kg/day, DM and CM rats respectively received 60 $\mu\text{g/ml}$ and 40 $\mu\text{g/ml}$ of melatonin in drinking water. Bottles containing melatonin solution were covered with aluminium foil as melatonin is light sensitive. The melatonin solution was stored at -4°C until used. Drinking water with or without melatonin was replaced every day.

To test the possible long term additional effects of the melatonin vehicle (0.05% ethanol), we conducted a separate study where animals were divided in four groups: C, control rats receiving drinking water only; CE, control rats receiving 0.05% (v/v) ethanol in drinking water; D diet induced obesity rats receiving drinking water only, and DE, diet induced obesity rats receiving 0.05% (v/v) ethanol in drinking water. Absolute ethanol (1 ml) was dissolved in 2 L drinking water to yield a final concentration of 0.05%. Drinking water with or without ethanol was replaced every day.

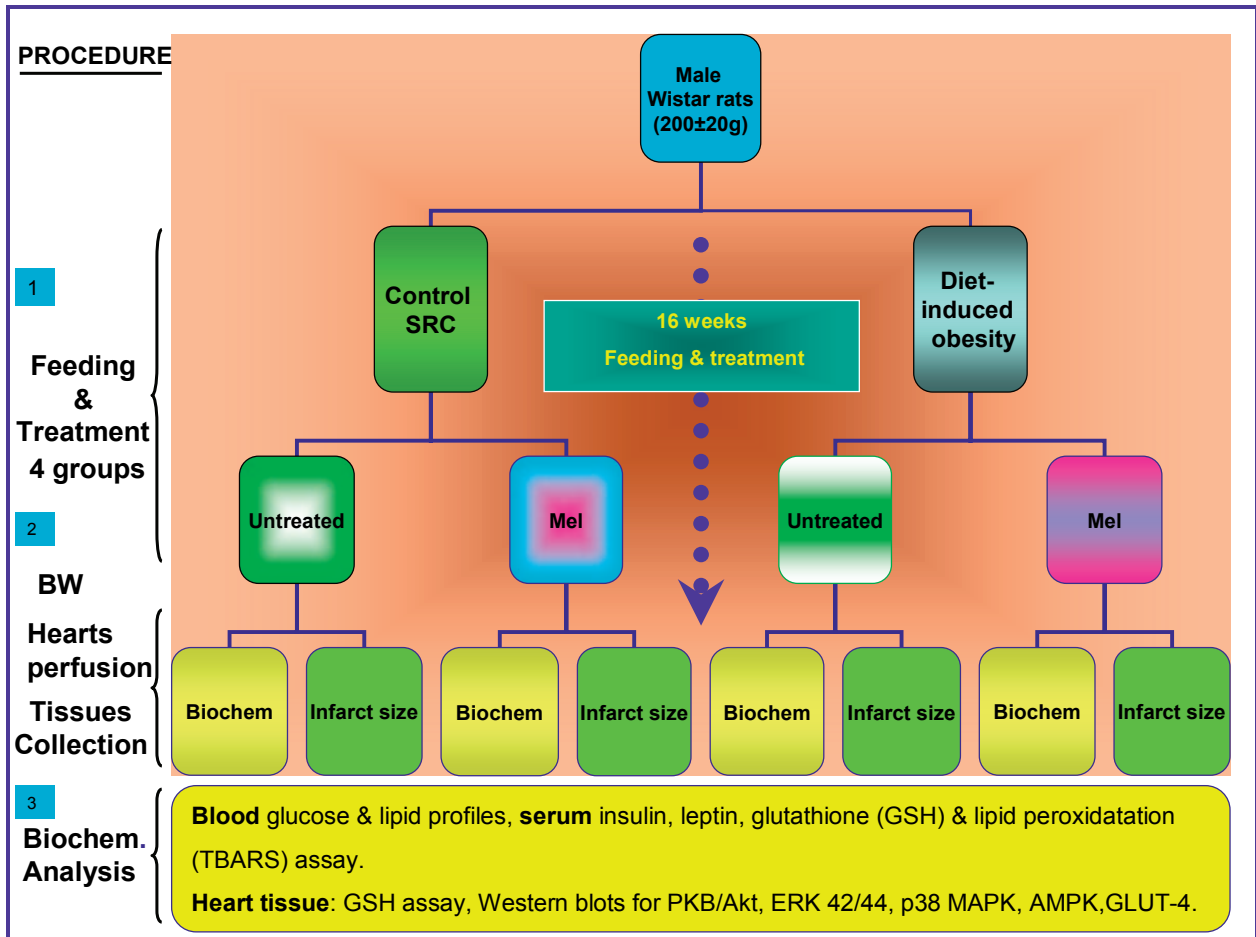


Figure 3- 1 Major phases of the study

(1): grouping, feeding and treatment; (2): determination of body and visceral fat weight, heart perfusion and collection of blood and heart tissue samples; (3): biochemical analyses. BW, body weight; Mel, melatonin; TBARS, thiobarbituric acid reactive substances; Biochem, biochemical analysis; SRC, standard rat chow. The arrow shows the time-direction of procedure.

3.3. EXPERIMENTAL PROCEDURE

The experimental procedures and major phases of the studies are presented in fig.3.1. After 16 weeks of feeding and treatment non-fasted or overnight fasted animals were sacrificed. Before the sacrifice, the animals were anaesthetized, and body weights determined. After collection of blood from the thoracic cavity of the rat, following the excision of the heart, visceral fat was collected and weighed. At the end of the reperfusion period of the isolated hearts, Evans blue suspension was infused (see section 3.3.1.5) and all excess non-cardiac

tissue removed, the hearts were rapidly blotted, weighed and subsequently frozen for infarct size determination. This technique has previously been used in other studies (Asai et al., 2005; Du Toit et al., 2005). The weight of the infused suspension was subtracted from the measured heart weight to obtain the final heart weight. In separate experiments, we found no differences between the weights of unperfused and reperfused hearts (after 2h reperfusion). The heart to body weight ratios were calculated and expressed in mg/g.

3.3.1. Heart perfusion

To perfuse the hearts a modified Krebs-Henseleit bicarbonate buffer (KHB) was used, containing (in mM): NaCl 119, NaHCO₃ 24.9, KCl 4.7, KH₂PO₄ 1.2, Mg SO₄.7H₂O 0.59, and Na₂SO₄ 0.59, CaCl₂.H₂O 1.25 and Glucose 10. The KHB was oxygenated and kept at pH 7.4 by gassing with 95% O₂ and 5% CO₂ at 37° C.

3.3.1.1. Isolated heart perfusion technique

After 16 weeks of feeding and treatment rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (Merck, Cape Town, South Africa) given at the dose of 30 mg/kg (Lochner et al., 2003). The rat heart was rapidly excised and arrested in ice-cold (4°C) normal saline solution and thereafter mounted *via* the aorta onto the aortic cannula as follows. A string was tied around the aorta to secure it firmly to the cannula and care was taken to avoid leaks between the aortic cannula and the aorta as well as air bubbles in the aortic cannula.

The hearts were first perfused in the Langendorff mode (retrogradely), in non re-circulating manner at a constant hydrostatic pressure (100 cm H₂O) for 15 minutes. This allows the hearts to stabilize as well as washout of blood. The left atrium was cannulated *via* the pulmonary vein. After 15 minutes of the retrograde perfusion, the perfusion mode was switched to the working heart mode (see perfusion protocol fig.3. 2) (preload 15 cm H₂O and afterload of 100 cm H₂O) (Neely et al., 1967; Depre, 1998). The subsequent contraction of the left ventricle expelled the perfusion solution to the aortic cannula against the constant pressure of 100 cm H₂O. The hearts were not electrically paced. Myocardial temperature was thermostatically controlled by inserting a temperature probe into the pulmonary artery. It was monitored at regular intervals (constant at 37°C during reperfusion and 36°5 C during ischaemia) and the water bath was adjusted accordingly. Two modes of ischaemia were used: regional and global ischaemia.

3.3.1.2. Induction of ischaemia

3.3.1.2.1. Regional ischaemia (fig.3.2 A)

After the stabilisation period, a silk suture with a ½ circle, 25 type needle size, was inserted underneath the proximal left anterior descending coronary artery (LAD) and after measuring coronary flow (CF), the suture was tightened and the LAD occluded for 40 minutes at 36.5°C. Adequate regional ischaemia was indicated by a 33% reduction in CF compared to the pre-ischaemic CF. Regional ischaemia was also confirmed by cyanosis of the myocardial surface. After 40 minutes of ischaemia, the silk suture was released, and the heart reperfused for 120 minutes to determine infarct size (see perfusion protocol fig. 3.2 A). Mechanical function was documented before induction of ischaemia and during reperfusion.

3.3.1.2.2. Global ischaemia (fig.3.2B)

The hearts were stabilized as mentioned above. After stabilisation, the aortic cannula was occluded causing a total cessation of coronary perfusion to the heart (no-flow ischaemia). During the ischaemic period, myocardial temperature was maintained at 36.5°C, and after 20 minutes of ischaemia, the aortic cannula was opened for reperfusion of the heart for 10 minutes (see perfusion protocol fig 3.2 B). At the end of reperfusion, hearts were freeze clamped and stored in liquid nitrogen for further biochemical analysis.

3.3.1.3. Perfusion protocol (fig.3.2)

Isolated hearts were perfused using two different protocols for the evaluation of myocardial function and infarct size as well as for biochemical analysis (western blot analysis) (see fig.3. 2). In the case of measurement of myocardial function and infarct size, as previously mentioned, regional ischaemia was induced for 40 minutes of coronary artery ligation followed by 120 minutes reperfusion (see fig.3.2 A), whereas for the evaluation of activation of pro-survival kinases (western blots), all hearts were subjected to a no-flow global ischaemia for 20 minutes (36°5C) followed by 10 minutes reperfusion (see fig.3.2 B). The pre-ischaemic function was documented after 15, and 30 minutes of stabilization (before ischaemia) and the post-ischaemic mechanical function was recorded at 30, and 60 minutes of reperfusion.

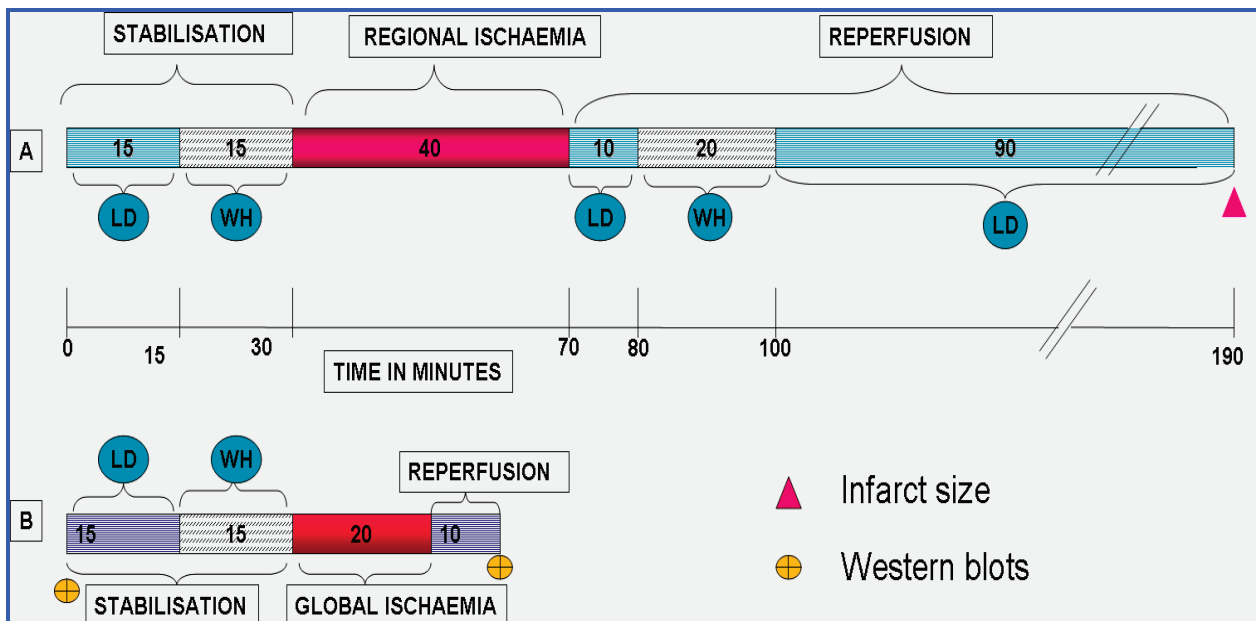


Figure 3- 2 Perfusion protocol.

A, determination of infarct size and myocardial function; B, western blot analysis (RISK pathway, p38 MAPK, AMPK and GLUT-4); LD, Langendorff; WH, working heart

3.3.1.4. Myocardial function determination

The coronary and aortic flow rates were measured manually. Cardiac output (CO) was calculated as the sum of aortic output (AO) and coronary flow (CF). The aortic systolic and diastolic pressure (mmHg) and heart rate (HR)(beats per minute (bpm)) were monitored and recorded on a computerized system through a side-arm of the aortic cannula connected to a Viggo-Spectramed pressure transducer coupled to the computer system. Total work performance (pressure power + kinetic power) developed by the heart was determined according to the formula described by Kannengieser et al. (1979). To determine functional recovery, the post-ischaemic value was expressed as the percentage of the pre-ischaemic value. All data were collected and analysed using a PhysiTutor® data acquisition system.

3.3.1.5. Determination of area at risk and infarct size

Myocardial infarct size was determined as previously described (Lochner et al., 2003). At the end of 2 hours reperfusion, the silk suture around the coronary artery was securely tied and

1ml of a 0.5% Evans blue suspension was slowly injected via the aorta cannula. The heart was then frozen overnight before being cut into 2 mm thick slices. After defrosting, the slices were stained with 1% w/v triphenyl tetrazolium chloride (TTC) in phosphate buffer pH 7.4 at 37°C for 15 minutes. Slices were then fixed in 10% v/v formaldehyde solution (at room temperature) to enhance the contrast between stained viable tissue and unstained necrotic tissue. At this stage, the damage for each slice was visible; the blue area indicating a viable tissue, the white or unstained area indicates a damaged or infarcted area surrounded by a red area. The red area together with the white area constitutes the area at risk. All slices were drawn and scanned. For each slice, the left ventricle area at risk (AR) and the area of infarcted tissue in the risk zone were determined using computerized planimetry (UTHCSA Image Tool programme, University of Texas Health Science Center at San Antonio, TX, USA). For each heart, the areas of the tissue slices were summated. The infarct size (IFS) was expressed as the percentage of the area at risk (I/AR %).

3.3.2. Biochemical analyses

3.3.2.1. Western blot analysis

3.3.2.1.1. Tissue collection

Hearts were either collected without perfusion (baseline) or after reperfusion (see protocol fig. 3.2 B). At baseline, hearts were excised and mounted on the perfusion apparatus as previously described. After flushing out of the blood and removing all surrounding tissues, the hearts were freeze-clamped with pre-cooled Wollen-berger tongs and plunged into liquid nitrogen for later biochemical analyses. To evaluate the post-ischaemic protein changes, hearts were freeze-clamped after reperfusion following global ischaemia. In a pilot study we found that the optimum activation of pro-survival proteins PKB /Akt, ERK 42 and 44 occurred during early reperfusion at 10 minutes (results not shown). Therefore, all hearts were routinely freeze-clamped at this time interval.

3.3.2.1.2. Preparation of lysates

Cytosolic PKB/Akt, ERK 42 and 44, p38 MAPK, AMPK, and GLUT-4 were extracted from frozen myocardial tissue. The tissue was pulverized and homogenized in 600 to 900µl of lysis buffer by a Polytron homogenizer. The lysis buffer was made up of (in mM) Tris-HCl 20, *p*-nitrophenylphosphate 20, EGTA 1.0, EDTA 1, NaCl 150, tetra-sodium-pyrophosphate 2.5, β-glycerophosphate 1.0, sodium orthovanadate 1.0, phenylmethyl sulphonyl fluoride (PMSF) 1.0, aprotinin 10 µg/ml and leupeptin 10 µg/ml, Triton-X100 1%, pH 7.4. Samples were then centrifuged at 1000xg for 10 minutes to obtain the supernatant, which was subsequently used for western blotting.

3.3.2.1.3. Western blot technique

The protein content for the expression of total and phosphorylated PKB/Akt, ERK; p38 MAPK, AMPK and GLUT-4 expression was determined using the Bradford technique (Bradford, 1976). The tissue lysates were then diluted in Laemmli sample buffer. They were boiled for 5 minutes and stored at -20°C.

3.3.2.1.3.1. Protein separation and transfer (tables 3.1 and 3. 2)

Depending on type of protein (see table 3.1), 10 to 50 µg of protein was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the standard BIO-RAD Mini-Protean III system. The gel was made up of a 10 or 12% polyacrylamide gel (resolving gel) and 4% stacking gel on the top of resolving gel (see gel composition table 3.2). The running buffer contained (in mM) Tris 250, glycine 192 and 1% sodium dodecyl sulphate (SDS). The electrophoresis separation technique is based on the mobility of ions in an electric field. Samples, including the commercial molecular weights marker, were loaded into wells in the gel. The marker helped to localize exactly the level of each protein separated, according to its molecular weight (see table 3.1). After separation, proteins were then transferred to a PVDF membrane (Immobilon™ P, Millipore) using a tank electrotransfer set up. The transfer buffer contained (in mM) Tris-HCl 25, glycine 192, and methanol (20% v/v). These membranes were routinely stained with Ponceau red for visualization of proteins and to confirm an adequate transfer.

3.3.2.1.3.2. Blocking of membrane and incubation with antibodies (table 3.1)

Non-specific binding sites on the membranes were blocked with 5% fat-free milk in TBST (Tris-buffered saline + 0.1% Tween 20) for 1 to 2 hours at room temperature and then incubated overnight at 4°C with the primary antibodies that recognize total or phospho-specific proteins. In this study we used total ERK p42/p44 and phospho-ERK p42/p44 (Thr-202/Tyr- 204); total PKB/Akt and phospho-PKB/Akt (Ser-473); total p38 MAPK and dual phospho-p38 MAPK (Thr-180/Tyr-182); total AMPK α and phospho-AMPK (Thr-172); and Glut-4 (purchased from Cell Signalling Technology). Primary antibodies were diluted in TBST solution or in 5% milk solution (see table 3.1). Membranes were washed with TBST (5x5 minutes) and then incubated for 1 hour at room temperature, with a diluted horseradish peroxidase-labelled secondary antibody (Amersham, LIFE SCIENCE). The secondary antibody was either diluted in TBST or in 5% fat free milk solution (see table 3.1).

3.3.2.1.3.3. Visualisation or immunodetection

After thorough washing with TBST, membranes were covered with ECL (enhanced chemiluminescence) detection reagents (Amersham, LIFE SCIENCE) and quickly exposed to an autoradiography film (Hyperfilm ECL, RPN 2103-Amersham, LIFE SCIENCE) to detect light emission through a non-radioactive method (ECL western blotting). Films were densitometrically analysed (UN-SCAN-IT, Silk Scientific Inc., Orem, Utah, USA) and activated protein values were determined. The protein activation was expressed as a ratio of phospho- to total protein.

Table 3- 1 Western blot analysis

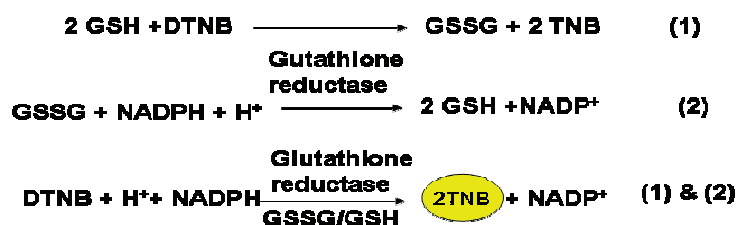
<i>Protein</i>	<i>Molecular weight</i>	<i>Protein loaded</i>	<i>Resolving gel (SDS-PAGE)</i>	<i>Primary antibody dilution</i>	<i>Secondary antibody dilution</i>
PKB/Akt	60kDa	30 μ g	12%	1:1000 TBST	1:4000 5% milk TBST
ERK 42/44	42/44kDa	25 μ g	12%	1:1000 TBST	1:4000 TBST
P38 MAPK	43kDa	25 μ g	12%	1:1000 TBST	1:4000 TBST
AMPK	62kDa	50 μ g	10%	1:1000 5% milk TBST	1:4000 5% milk TBST
Glut-4	45kDa	30 μ g	10%	1:1000 in TBST	1:4000 in TBST

Table 3- 2 SDS-polyacrylamide gel

<i>Reagent</i>	<i>Stock</i>	<i>10% Gel</i>	<i>12% Gel</i>	<i>4% Stack gel</i>
dH ₂ O	distilled	4.9 ml	3.35 ml	3.05 ml
Tris-HCl(pH 8.8)	1.5 M	2.50 ml	2.50 ml (1.5M)	———
Tris-HCl(pH 8.8)	0.5 M	———	———	1.25 ml
SDS	10%	100 µl	100 µl	50 µl
Acrylamide	40%	2.50 ml	3.0 ml	0.5 ml
APS	10%	50 µl	50 µl	50 µl
TEMED	99%	20 µl	20 µl	10 µl

3.3.2.2. Glutathione assay

To determine the myocardial antioxidant status, the levels of total glutathione were determined using the Glutathione Assay Kit purchased from Sigma Aldrich (St Louis, MO, USA). The total glutathione is the sum of the reduced glutathione (γ -glutamylcysteinylglycine) (GSH)(90-95%) and glutathione disulfide (GSSG) (oxidized glutathione) (5-10%). GSH is the key antioxidant in animal tissue. All reagents were prepared according to the technical bulletin accompanying the assay kit. Here we described briefly the assay principle and procedure (for assay details see Akerboom & Sies, 1981). The principle of this assay is the reaction in which catalytic amounts of GSH cause a continuous reduction of 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB) (see below reaction 1). The GSSG formed is then recycled by glutathione reductase and NADPH to generate GSH (see reaction 2). The principle is summarized in the following reactions (1 & 2).



Frozen tissue was pulverized and an aliquot (0.1-0.3 g) was mixed and homogenised with 10 volumes of 5% 5-sulfosalicylic acid (SSA) to remove proteins. After 10 minutes at 4°C, the suspension was centrifuged at 10,000xg for 10 minutes. The supernatant was measured and kept at -80° C as the original sample. Additional dilutions were made to obtain a sample

solution in the detection range. To 10 µl of sample, 150 µl of working mixture (95mM potassium phosphate buffer pH7.0, 0.95mM EDTA, 0.031 mg/ml 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB), and 0.115 units /ml glutathione reductase) was added. The mixture was then incubated at room temperature for 5 minutes and 50 µl of the diluted NADPH solution (48 µM NADPH) was added. The mixture became yellowish as the final product 5-thio-2-nitrobenzoic acid (TNB) is yellow. This was measured spectrometrically at 412 nm using a plate reader. A standard curve was used to determine the amount of glutathione. GSH content in unknown sample was calculated using the following formula:

$$\frac{\text{nmoles of GSH per ml of sample}}{1} = \frac{\Delta A_{412} / \text{min}(\text{sample}) \times \text{dil}}{\Delta A_{412} / \text{min} (1 \text{ nmole}) \times \text{vol}}$$

$\Delta A_{412}/\text{min} (\text{sample})$ = slope generated by the samples after subtracting the values generated by the blank reaction; $\Delta A_{412}/\text{min} (1\text{nmole})$ = slope calculated from standard curve for 1 nmole of GSH; dil. = dilution factor of original sample; vol. = volume of sample in the reaction in ml.

The final level of GSH was expressed in nmoles /gram of wet tissue. The same procedure was used for determination of serum GSH with some differences in sample extraction and calculation of the final GSH concentration. 200µl of serum was used as original sample in place of supernatant and the final levels of GSH was expressed in nmoles / ml of serum.

3.3.2.3. Blood analysis

3.3.2.3.1. Blood collection

Blood samples were collected as previously described (Du Toit et al., 2005). Blood was obtained from the thoracic cavity immediately after removal of the heart and collected in serum separation tubes (BD Vacutainer). Tubes were centrifuged at 3,000 rpm at 4°C within 30 minutes of collection. Serum was transferred in eppendorff tubes and stored at -80°C for further analyses.

3.3.2.3.2. Blood glucose determination

Fasting blood glucose was measured using the glucose meter (Gluco Plus™, distributed by CIPLA DIBCARE, Bellville, South Africa). A small drop of flesh blood, obtained by pricking the

distal tail with a lancet was placed on a disposable test strip and after 5 seconds, the meter displayed the blood glucose level expressed in mmol/l.

3.3.2.3.3. Blood lipid levels

Fasting lipid levels were determined using CardioCheck[®] PA analyser according to the instructions sheet accompanying the test strips. After the removal of the heart, 40 µl of flesh blood was collected and placed on a lipid test strip (PTS Panels[™]; Polymer technology, IN, USA). After 2 minutes the levels (in mmol/L) of total cholesterol, HDL-cholesterol, triglycerides were displayed. Serum LDL cholesterol was not measured as this was not possible using this test.

3.3.2.3.4. Insulin assay

Fasting serum was collected as described above. The insulin level was determined using a competitive radioimmunoassay (RIA) (Coat-A-Count[®] Insulin Diagnostic Products Corporation; LA, USA).

3.3.2.3.4.1. Radioimmunoassay principle

A fixed concentration of labeled tracer antigen is incubated with a constant dilution of antiserum so that the concentration of antigen binding to sites on the antibody is limited. For example only 50% of the total tracer concentration may be bound by antibody. If unlabeled antigen is added to this system, there is competition between the labeled tracer and the unlabeled antigen for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can then be measured after separating the free and bound antibodies.

In the case of insulin test, a fixed amount of labelled insulin would compete with insulin present in blood samples for binding sites on an insulin specific antibody. The antibody is immobilized in the wall of the tube. The free and bound antibodies are separated by decanting the tubes. The radioactivity was measured using a gamma scintillation counter (Cobra II Auto Gamma, ADP, South Africa).

3.3.2.3.4.2. Assay procedure

Before the assay was performed, the components were brought to room temperature. All samples were done in duplicate. Uncoated polypropylene tubes were labeled for total counts (T) and non specific binding (NSB). Insulin antibody coated tubes were labeled for standards, controls and serum samples. 200 µl of the zero calibrator A was pipetted into the NSB tubes. 200 µl of the remaining calibrator, control and sample were pipetted in their respective tubes. 1.0 ml of ¹²⁵I insulin was added to each tube and subsequently vortexed and incubated for 22-24 hours at room temperature. The tubes were then decanted (except for the total counts) and the samples allowed to drain for 2-3 minutes. Excess liquid was blotted from the lip of tubes and the radioactivity for each tube was determined in gamma counter for 1 minute. A standard curve was included to determine the concentration of the unknown samples.

The homeostasis model assessment (HOMA) index was calculated as the product of fasting glucose concentration (mmol/l) multiplied by insulin concentration (µU/ml) divided by 22.5 (Muniyappa et al., 2008).

3.3.2.3.5. Leptin assay

Serum was collected as previous mentioned. Serum leptin levels were measured using a rat leptin radioimmunoassay (RIA) kit (Linco Research, St. Charles, MO, USA) according to information product accompanying the kit. The RIA principle is described above. Briefly, assay buffer was pipetted (300 µl) in non specific binding (NSB) tubes, (200 µl) in the reference (Bo) tubes, and (100 µl) in standards or quality controls or samples tubes. 100 µL of Standards (0.5, 1, 2, 5, 10, 20, and 50 ng/L) or quality controls or samples were respectively added to their assay buffer tubes. The anti-leptin antibody (100 µL) was added and in all tubes except the total and NSB tubes, mixed and incubated overnight (24 hours) at room temperature. 100 µL of ¹²⁵I-Rat leptin tracer was added and mixed to all tubes, and incubated for an additional 24hours. A cold precipitating reagent (1.0 mL; anti-guinea pig IgG, raised in goats) was added to all tubes except the total count tubes and incubated for 20 min at 4°C to precipitate the antibody/leptin complex. All tubes were then centrifuged for 20 minutes at 3000xg at 4°C. The supernatants were decanted and the pellet radioactivity was counted using a gamma counter (Cobra II Auto Gamma; ADP, South Africa). The leptin concentrations for the samples were calculated from the standard curve and expressed in ng/ml.

3.3.2.3.6. Lipid assay

Lipid peroxidation refers to the oxidative degradation of lipids. Non fasting serum samples were assayed for lipid peroxidation, total cholesterol (TC), triglycerides (TRIG) and phospholipids (PL). Serum TC and TRIG concentrations were determined using enzymatic colorimetric kits (KAT Medicals, Calicom Trading (PTY), South Africa) and PL (WAKO Chemicals, Germany) by a Labsystems Multiskan MS analyzer (AEC Amersham Co., South Africa). TRIG and PL were determined by the same methods, for standardization as reference mass for lipid peroxidation.

Concentrations of conjugate dienes (CD), lipid hydroperoxide (LOOH) and thiobarbituric acid reactive substances (TBARS) were measured in non fasting serum by spectrophotometric methods using a GBC UV/VIS analyser (Wirsam Scientific and Precision Equipment, South Africa) (CD) or Labsystems Multiskan MS Analyser (AEC Amersham Co., South Africa) (LOOH and TBARS). Concentrations were calculated using the appropriate molar extinction coefficients and standards.

CD were measured at 234 nm after appropriate dilution in cyclohexane (Spectrosol) (Esterbauer et al., 1989; Pryor & Castle, 1984). After mixing the serum samples with cyclohexane, separation was enhanced by centrifugation (14,000 × *g* for 10 min at 10°C).

To determine LOOH, serum was assayed in the presence of xylenol orange (3,3'-bis[*N,N*-di(carboxymethyl)-aminomethyl]-*o*-cresolsulfonephthalein) (sodium salt) (Sigma Chemical Co., St Louis, MI, USA) and Fe²⁺ in the ferrous oxidation/xylenol orange (FOX) assay that was adapted to enhance the solubility of non-polar compounds by including chloroform. Absorbance of the resulting Fe³⁺-xylenol orange complex was measured at 560 nm (Jiang et al., 1991 & 2002).

TBARS were measured according to the method of Jentzsch et al. (1996). The samples (200 µl) were mixed with 25 µl butylated hydroxytoluene (BHT) (Fluka Chemie, Switzerland) in ethanol (Merck Chemicals, South Africa) and orthophosphoric acid (Sigma) buffer at pH 3.6 and vortexed for 10 seconds. 25 µl thiobarbituric acid (TBA) (Sigma) reagent was added and vortexed again. After incubation at 90°C for 45 minutes in water bath, the reaction was stopped by putting tubes on ice. TBARS were extracted with *n*-butanol. Saturated NaCl was added and the mixture was then centrifuged at 12,000 rpm for 1 minute. Absorbance was read at 532 nm.

3.4. DATA ANALYSIS

All data were expressed as mean \pm standard error of the mean (SEM). Infarct size was expressed as a percentage of the area at risk, and for functional recovery data, reperfusion aortic output and work total were expressed as a percentage of the pre-ischæmic value. When comparisons between two groups (diet and control groups) were made, an unpaired Student t-test was performed. For multiple comparisons, the ANOVA (two-way when appropriate) followed by the Bonferroni correction was applied. A p-value of <0.05 was considered significant.

CHAPTER FOUR

RESULTS

4.1. BIOMETRIC AND METABOLIC DATA

4.1.1.Characteristics of the diet-induced obesity model (fig.4.1)

The body weight of control (C) rats was averaged 387.8 ± 11.3 g. The high calorie diet (D) increased the body weight by 29.6% ($p < 0.001$) over a period of 16 weeks (fig.4.1A). It also increased the visceral fat content by 78.1% ($p < 0.001$). Fasted blood glucose (fig.4.8) was not different between the control (C) and high calorie diet (D) groups. Serum insulin and leptin levels were elevated by 80.6% ($p < 0.05$) and 80.7% ($p < 0.05$) respectively in the diet fed (D) rats (fig.4.1). Lipid profiles were also affected. Compared to control rats, the high calorie diet caused a significance increase in triglyceride (1.44 ± 0.1 vs 0.87 ± 0.1 ; $p < 0.01$) and a reduction in high density lipoprotein (HDL)-cholesterol (0.67 ± 0.1 vs 0.92 ± 0.1 ; $p < 0.01$) (fig.4.1). The heart weight of control rats was 1013 ± 44 mg. The high calorie diet increased the heart weight by 29.7% ($p < 0.05$) (fig.4.6). The heart weight to body weight ratio was 2.58 ± 0.1 mg/g for control rats and the diet had no significant effects on the heart weight/body weight ratio (fig.4.7).

4.1.2. Impact of the melatonin vehicle (0.05% v/v ethanol)

Melatonin was dissolved in small amount of ethanol and added in drinking water to make the final solution of 0.05% (v/v) ethanol in drinking water. To test the possible long-term additional effects of the vehicle (0.05% (v/v) ethanol), ethanol was added to the drinking water and its effects documented. The impact of vehicle on body weight (BW) and metabolic profile is presented in figures 4.2 and 4.3. Compared to drinking water alone, the administration of 0.05%v/v ethanol to drinking water for 16 weeks did not have any effect on body weight gain (fig.4.2). BW was 356.6 ± 20.6 g in control rats receiving 0.05% ethanol (CE) and 349.3 ± 11.48 g in controls receiving drinking water only (C). BW was 416.4 ± 21.6 g for diet with ethanol (DE) and 446 ± 29.1 g for diet with drinking water only (D) (fig.4.2).

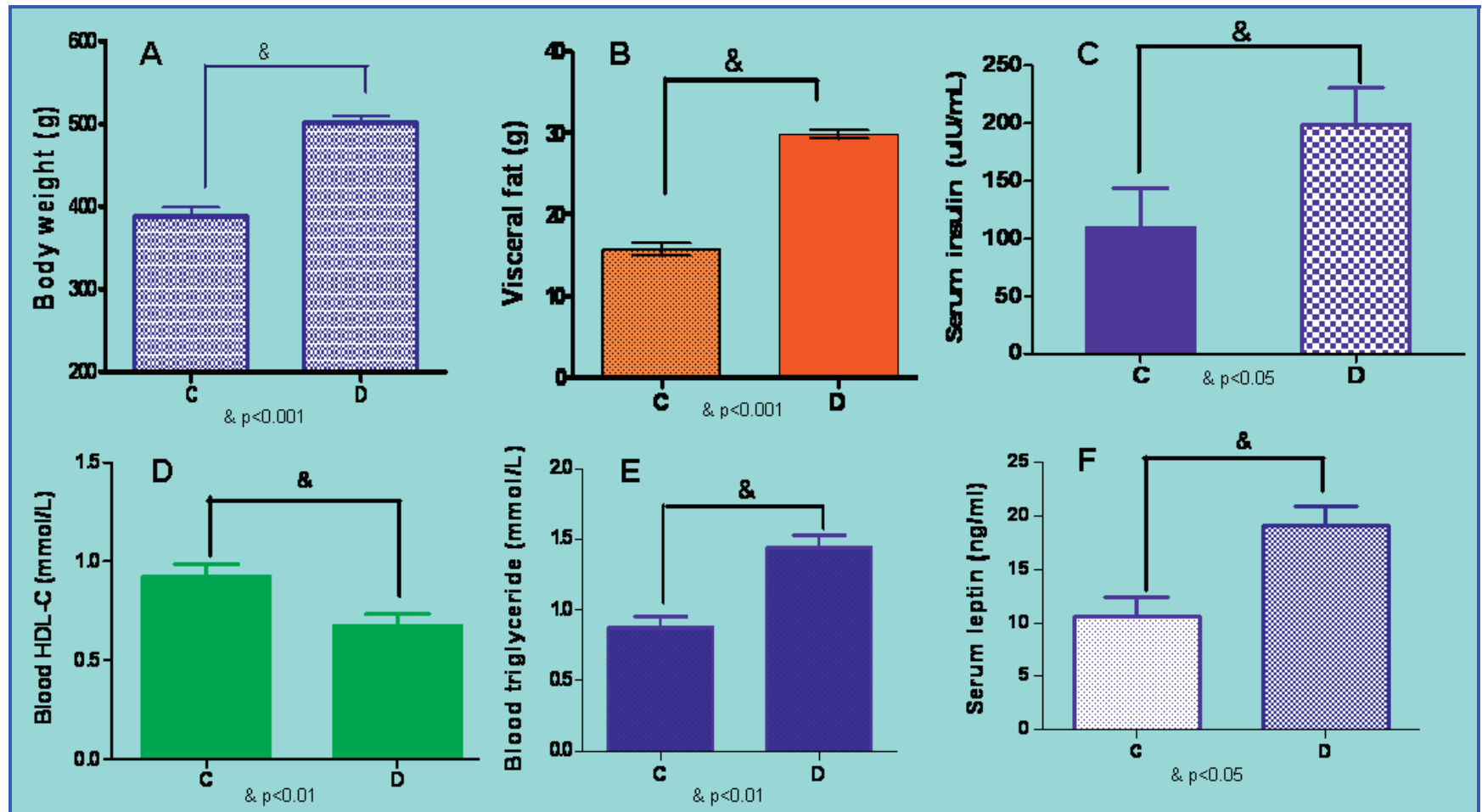


Figure 4- 1 Characteristics of our model Effects of diet on (A) body weight (n=14 -15), (B) visceral fat (n=11), (C) serum insulin (n=6), blood (D) HDL-cholesterol (n=6), (E) triglyceride (n=6), and (F) leptin (n=6). C, control; D, high calorie diet. &: p value (C vs. D).

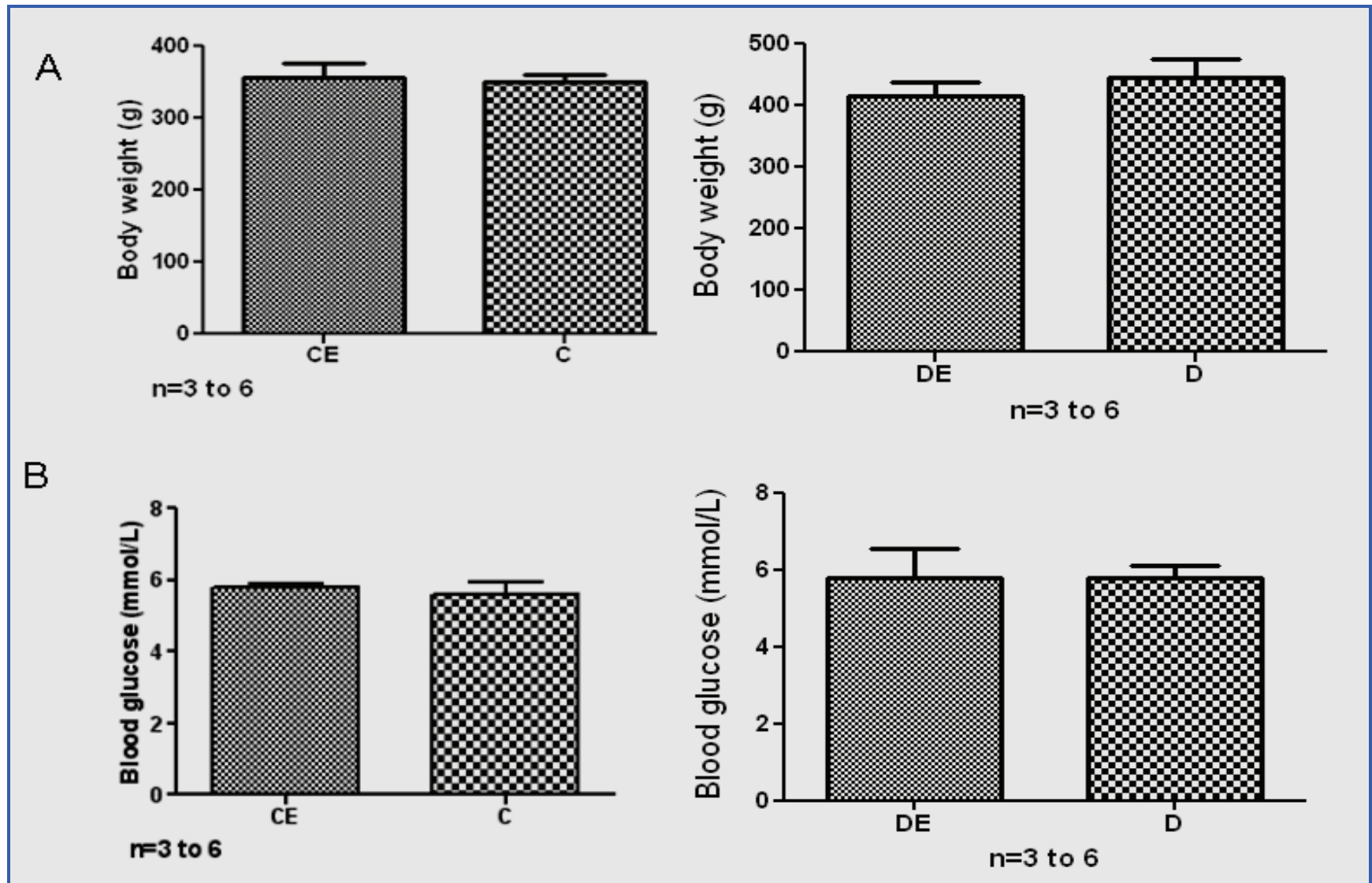


Figure 4- 2 Impact of vehicle on body weight (A) and fasted blood glucose (B).

CE, control + ethanol; C, control; DE, diet + ethanol; D, high calorie diet. n=3 to 6, $p > 0.05$ (ethanol vs. drinking water).

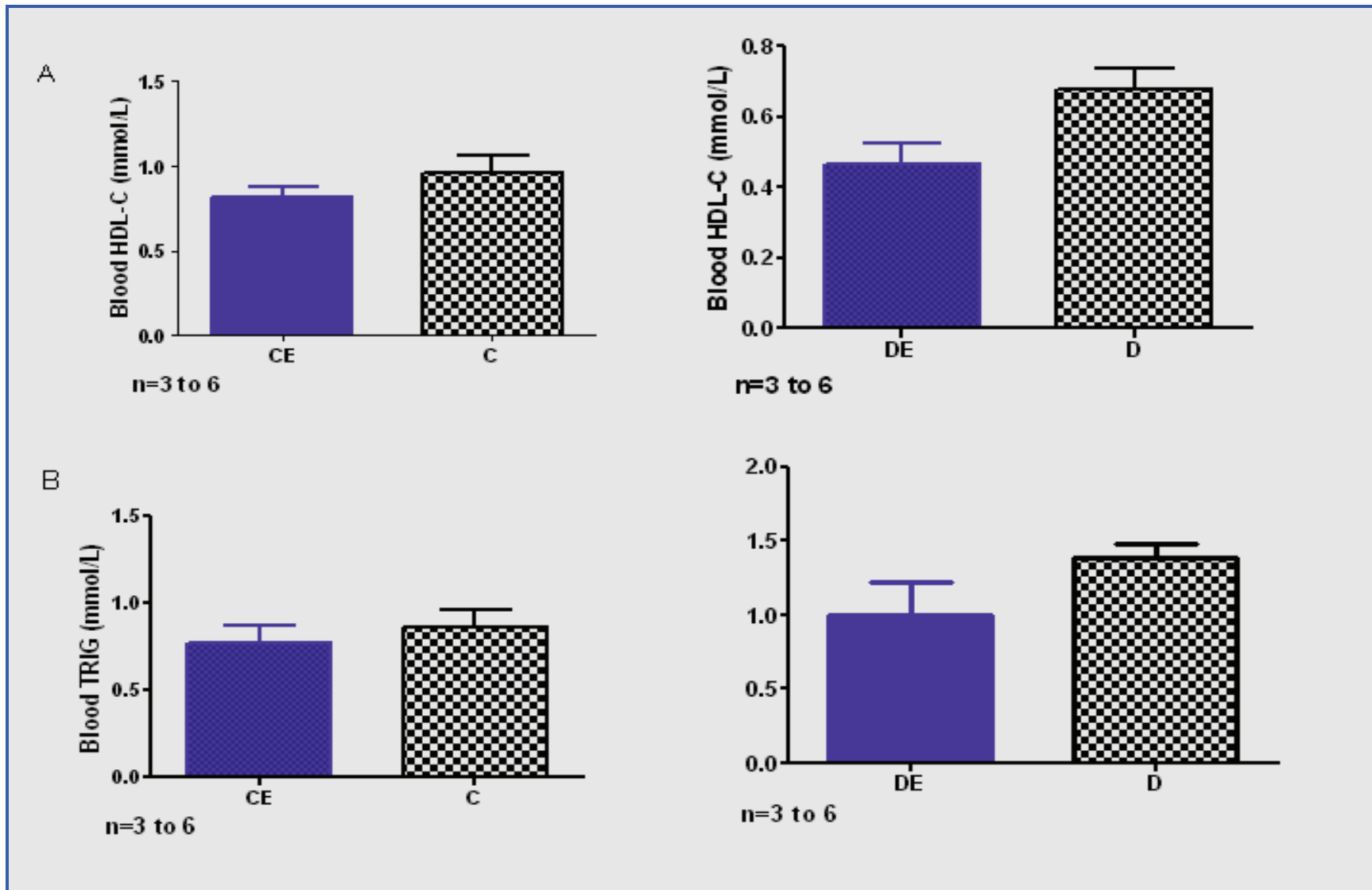


Figure 4- 3 Impact of vehicle on fasted blood lipid profile: (A) HDL-C, (B) TRIG (triglycerides).

CE, control + ethanol; C, control; DE, diet + ethanol; D, high calorie diet; n=3 to 6, p >0.05 (ethanol vs. drinking water).

4.1.3. Effects of chronic melatonin treatment

4.1.3.1. Biometric parameters

Chronic melatonin administration significantly reduced body weight gain by 10.7% in control (347.8 ± 9 vs 387.8 ± 11 g, $p < 0.05$) and 8.1% in diet (461.1 ± 11.5 vs 501.7 ± 8.1 g, $p < 0.05$) groups (fig. 4. 4). Melatonin also reduced visceral adiposity in control (12.6 ± 0.9 vs 15.7 ± 0.7 g, $p < 0.05$) and in diet (25.8 ± 1.1 vs 29.8 ± 1.1 g, $p < 0.01$) groups. Heart weight was reduced in both control (21%, $p < 0.05$) and diet (29.2%, $p < 0.01$) rats. Heart weight / body weight ratio was also lowered in both control (2.16 ± 0.1 vs 2.58 ; $p < 0.05$) and in diet (1.97 vs 2.62 ± 0.1 , $p < 0.01$) groups. The effects of chronic melatonin administration on biometric parameters are presented in figures 4. 4-7 and table 4.1

Table 4- 1 Biometric parameters

Parameters	<i>C</i>	<i>CM</i>	<i>D</i>	<i>DM</i>	<i>N</i>
Body weight (g)	387.8 ± 11.2	347.0 ± 9.7	501.7 ± 8.1	461.1 ± 11.1	14-15
Absolute heart weight (mg)	1013 ± 44.0	800.0 ± 68.8	1314 ± 19.9	930.0 ± 41.6	8-10
H W/BW ratio (mg/g)	2.58 ± 0.1	2.16 ± 0.1	2.62 ± 0.1	1.97 ± 0.1	8-10
Visceral adiposity (g)	15.7 ± 0.7	12.6 ± 0.9	29.87 ± 0.4	25.8 ± 1.1	11

HW/BW, heart weight to body weight ratio. *C*, control; *CM*, control with melatonin; *D*, high calorie diet; *DM*, high calorie diet with melatonin.

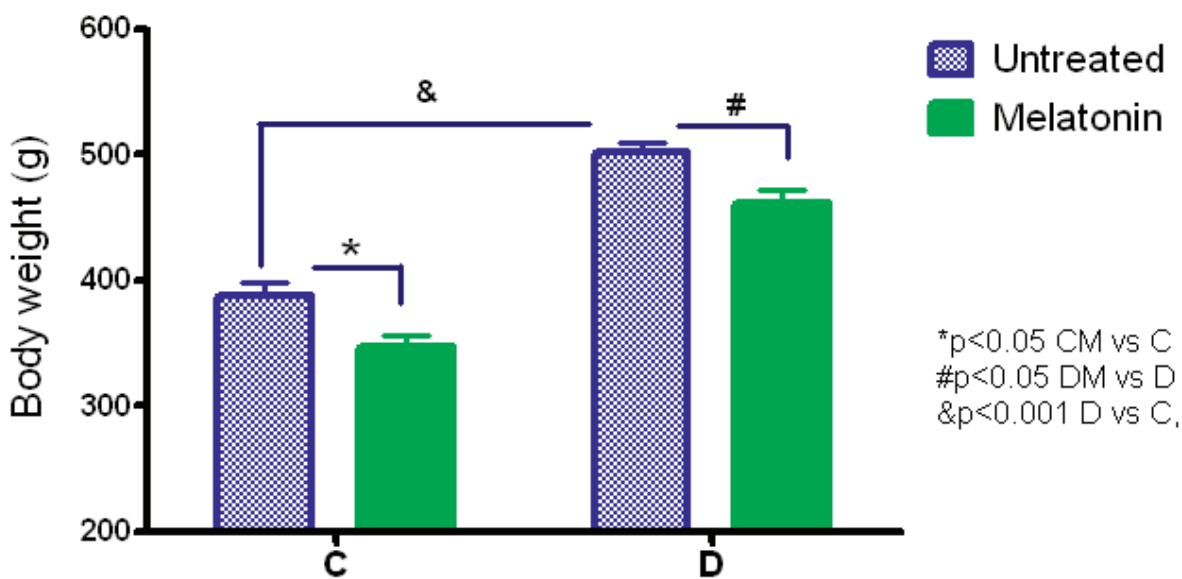


Figure 4- 4 Body weight (n= 14-15/group).

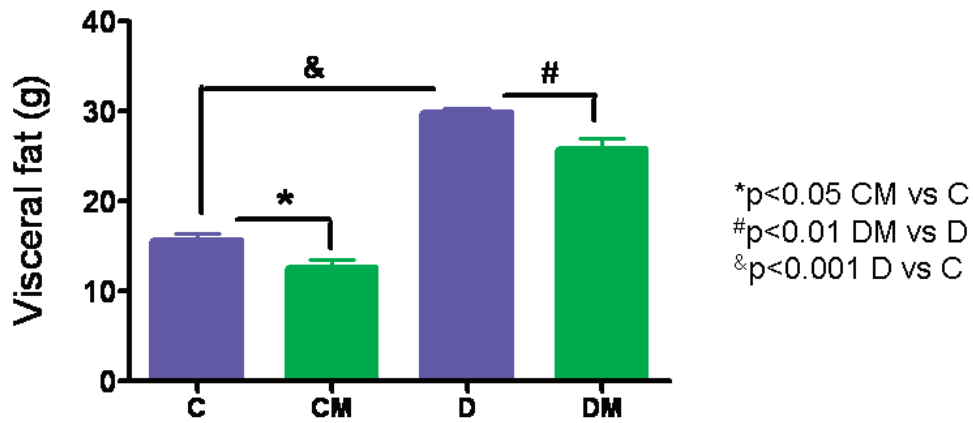


Figure 4- 5 Visceral fat (n=11/group).

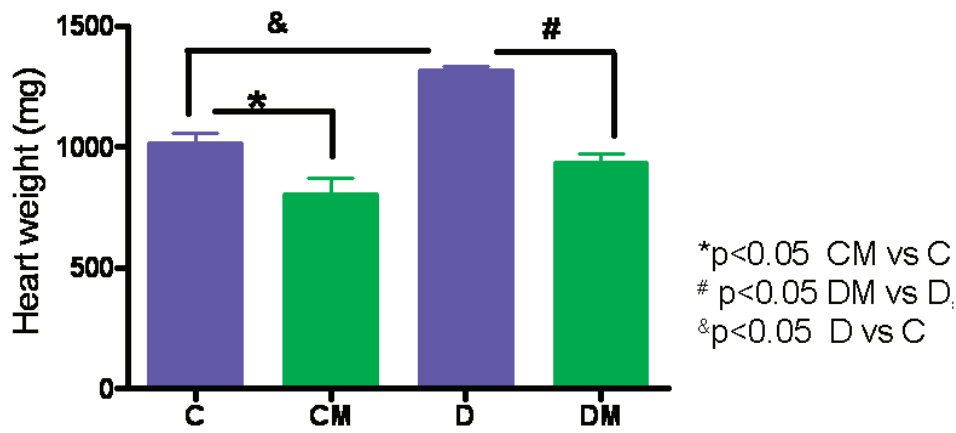


Figure 4- 6 Absolute heart weight (n=8-10/group).

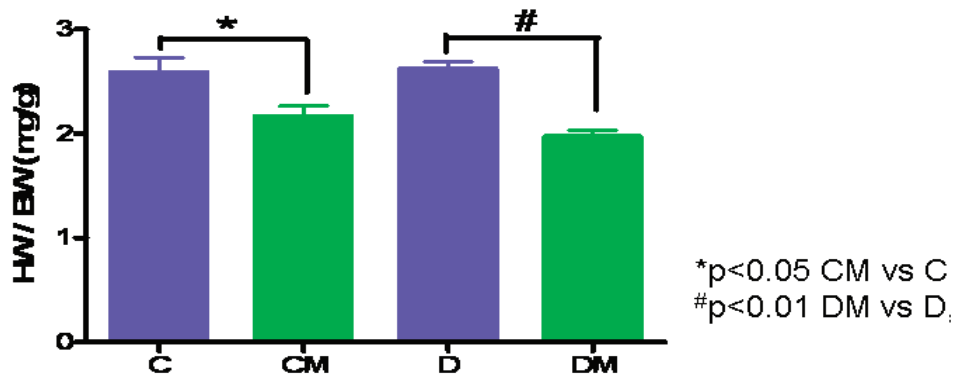


Figure 4- 7 Heart weight / body weight (HW/BW) ratio (n=8-10/group)

4.1.3.2. Metabolic data

The effects of chronic melatonin administration on metabolic parameters are presented in figures 4.8-13 and table 4.2. Fasted blood glucose levels of control (C) and diet (D) rats were 5.6 ± 0.2 and 5.71 ± 0.4 mmol/L, respectively. Melatonin treatment had no significant effects on fasted glucose levels of either control or diet groups (fig.4.8). Overnight fasted insulin levels were significantly reduced in the melatonin treated diet rats (61.3 ± 13.3 vs. 198.3 ± 32.2 μ IU/mL, $p < 0.05$) while there was no significant effect of melatonin on insulin levels in control rats (fig.4.9). Accordingly, the homeostasis model assessment (HOMA) index was reduced by 70.7 % ($p < 0.01$) in diet treated (DM) group, while there was no significant difference between HOMA index of treated and untreated control rats (fig.4.10).

Serum leptin levels were also reduced in diet treated (DM) rats by 52.6 % ($p < 0.01$) while there was no significant difference between leptin levels of treated and untreated control (CM and C) rats (fig.4.11). Blood lipid profiles were also affected by melatonin treatment. Triglyceride levels were 1.44 ± 0.1 mmol/L in diet and 0.87 ± 0.1 mmol/L in control untreated (C) rats. HDL-cholesterol levels were 0.67 ± 0.06 and 0.92 ± 0.06 mmol/L in diet (D) and control (C) groups, respectively. Melatonin administration reduced triglycerides and increased HDL-cholesterol levels by 25% ($p < 0.05$) and 29.8% ($p < 0.05$), respectively in diet fed rats (DM vs D) while having no effect in control (CM vs C) groups (fig.4.12 & 4.13).

Table 4- 2 Metabolic parameters

Parameters	C	CM	D	DM
Serum insulin(μ IU/mL)	109.8 \pm 33.9	68.1 \pm 12.6	198.3 \pm 32.2	61.3 \pm 13.4
Glucose (mmol/L)	5.6 \pm 0.2	5.2 \pm 0.3	5.7 \pm 0.4	5.3 \pm 0.5
HOMA index	27.48 \pm 8.4	14.16 \pm 1.7	47.03 \pm 5.8	13.75 \pm 2.8
Serum leptin(ng/mL)	10.56 \pm 1.8	10.62 \pm 2.8	19.09 \pm 1.8	9.06 \pm 1.5
HDL-C (mmol/L)	0.92 \pm 0.06	1.04 \pm 0.03	0.67 \pm 0.06	0.87 \pm 0.06
TRIG (mmol/L)	0.87 \pm 0.08	0.72 \pm 0.05	1.44 \pm 0.09	1.08 \pm 0.06

C, control; CM, control with melatonin; D, high calorie diet, DM, high calorie diet with melatonin; HOMA, homeostasis model assessment; TRIG, triglyceride, n=6/group.

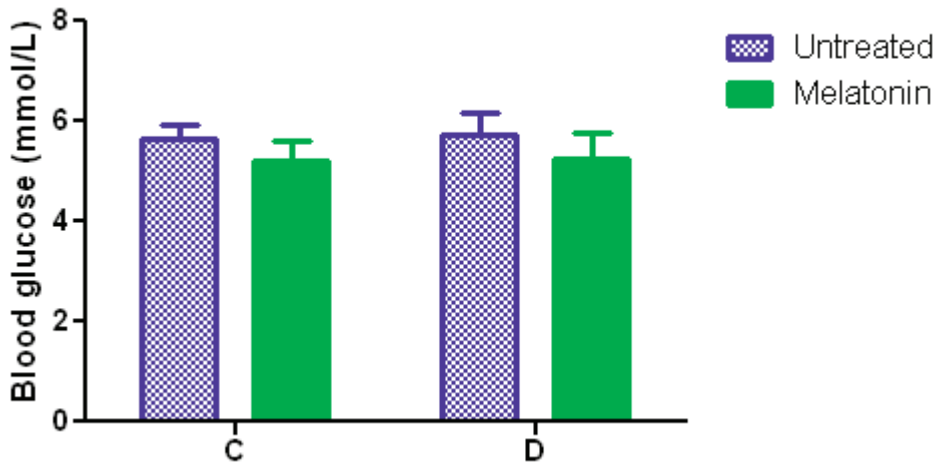


Figure 4- 8 Effect on fasted serum blood glucose (n=6/group)

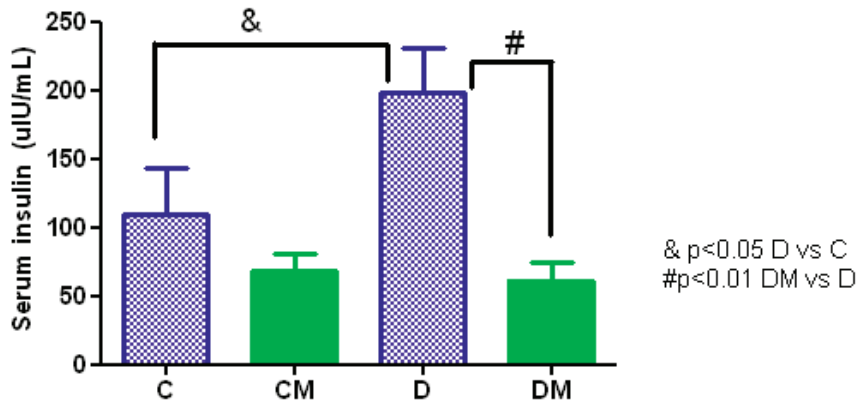


Figure 4- 9 Effect on fasted serum insulin (n=6/group)

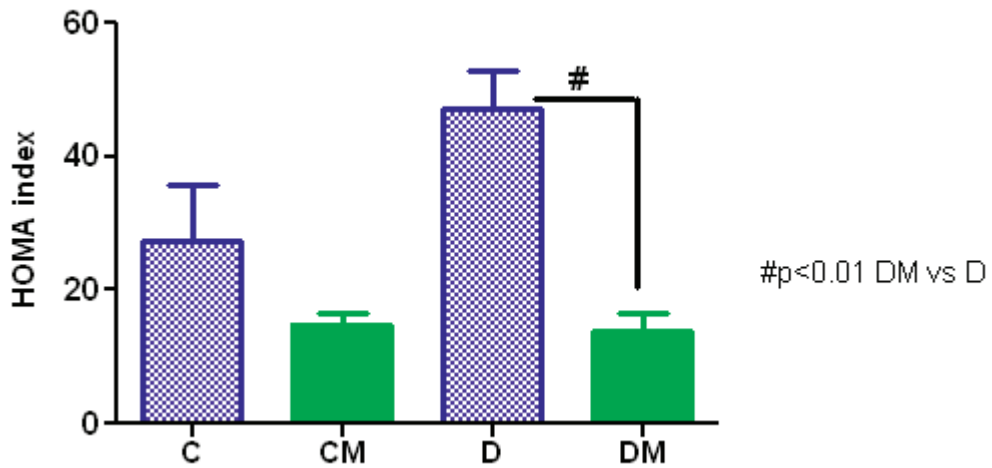


Figure 4- 10 Homeostasis model assessment (HOMA) index (n=6/group)

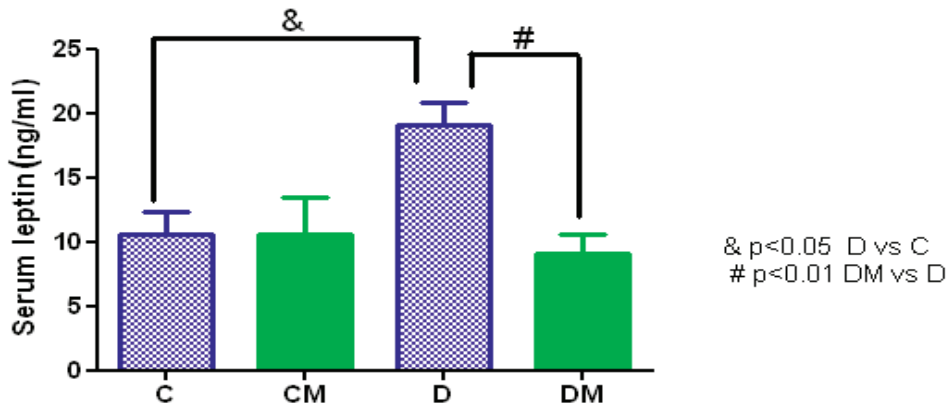


Figure 4- 11 Serum leptin (non-fasted) (n=6/group).

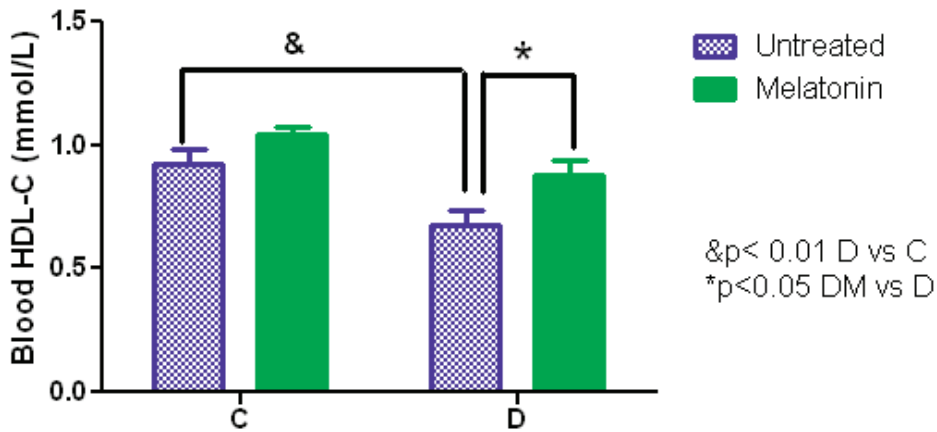


Figure 4 12 Fasted blood HDL-Cholesterol (n = 6/group)

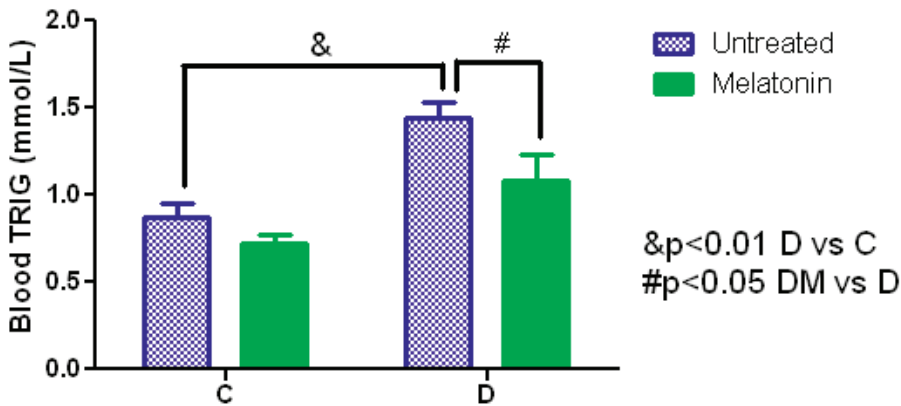


Figure 4- 13 Fasted blood triglycerides (TRIG) (n = 6/group).

4.2. MYOCARDIAL FUNCTION DATA

Chronic melatonin treatment had no significant effect on pre-ischaemic functional parameters (coronary flow, aortic output, work total, systolic and diastolic pressure, heart rate) of the isolated rat hearts from the control (table 4. 3). It increased however the aortic output and work total in diet fed rats (table 4. 3).

After coronary artery occlusion and 30 minutes of reperfusion hearts from high calorie diet fed rats were more prone to myocardial dysfunction compared to the control group. Percentage aortic output (AO) recoveries were ($6.2 \pm 6.2\%$ vs $46.8 \pm 4.6\%$, $p < 0.001$) and work total (WT) ($20.4 \pm 7.9\%$ vs $48.6 \pm 3.9\%$, $p < 0.001$) (fig.4.14 & 4.16).

In diet fed rats, chronic melatonin treatment increased percent recovery of aortic output ($28.5 \pm 6.5\%$ for DM vs. $6.2 \pm 6.2\%$ for D, $p < 0.05$) and work total ($34.5 \pm 5.6\%$ for DM vs $20.4 \pm 7.9\%$ for D, $p < 0.05$) (fig.4.14 & 4.16). Coronary flow recovery was also increased in treated compared to untreated high calorie diet hearts ($81.2 \pm 4.5\%$ vs $66.5 \pm 5.1\%$, $p < 0.05$). Accordingly, cardiac output was increased by 60% in treated compared to untreated high calorie diet groups ($44.4 \pm 5.2\%$ for DM vs 26.6 ± 5.1 for D, $p < 0.05$) (fig.4.15 and table 4.3).

At 60 minutes of reperfusion, retrograde perfusion mode was converted to working heart mode for 5 minutes to record the functional parameters at this point. As at 30 minutes of reperfusion, CO values continued to be elevated (by 68.7%) in treated compared to untreated high calorie diet fed hearts (DM vs D) while there was no significant difference in control rats (CM vs C). (tables 4.3 & 4. 4; fig.4.17). Accordingly, the percentage CO recovery was also increased in treated compared to untreated hearts ($25.9 \pm 4.9\%$ (D) vs $37.4 \pm 6.2\%$ (DM), $p < 0.05$) The effects of diet and chronic melatonin administration on isolated rat heart function are presented in tables 4.3 – 4.4 and figures 4.14-4.17.

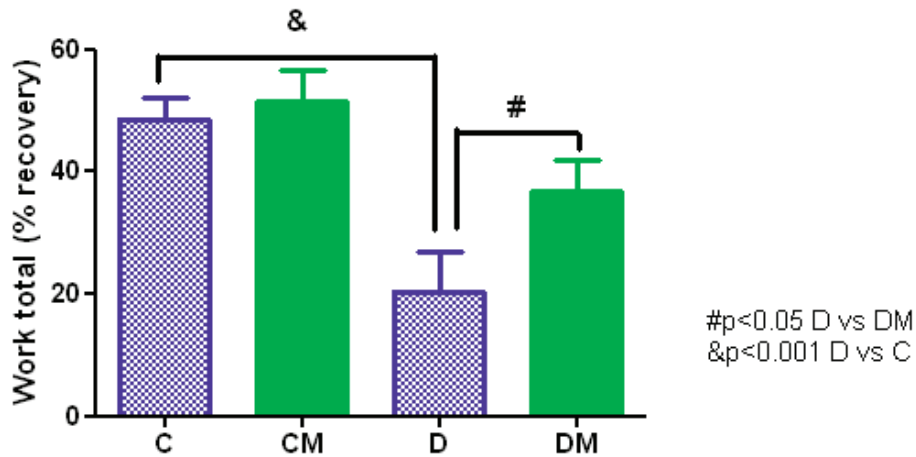


Figure 4- 14 Percentage work total recovery after 30 minutes of reperfusion (n=8-10/group)

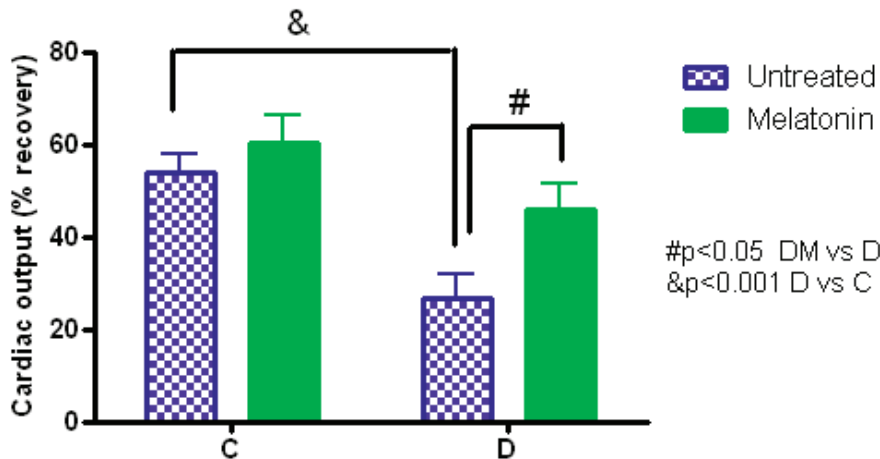


Figure 4- 15 Percentage cardiac output recovery after 30 minutes of reperfusion (n=8-10/group).

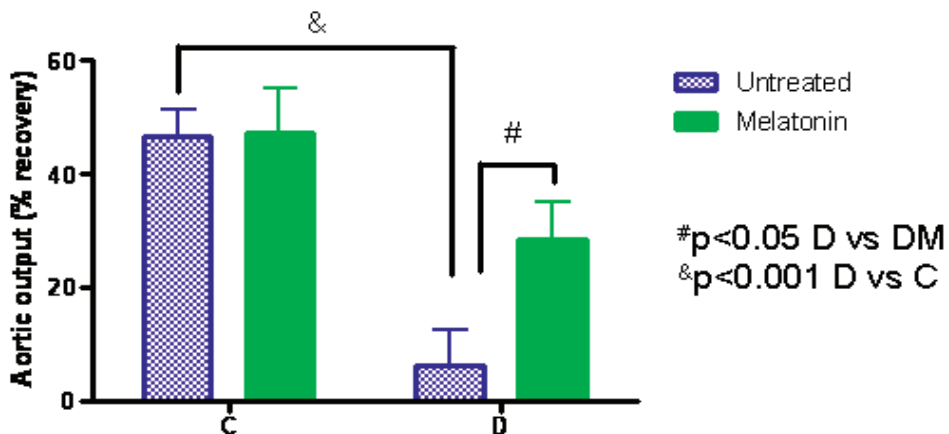


Figure 4- 16 Percentage aortic output recovery after 30 minutes reperfusion (n=8-10/group)

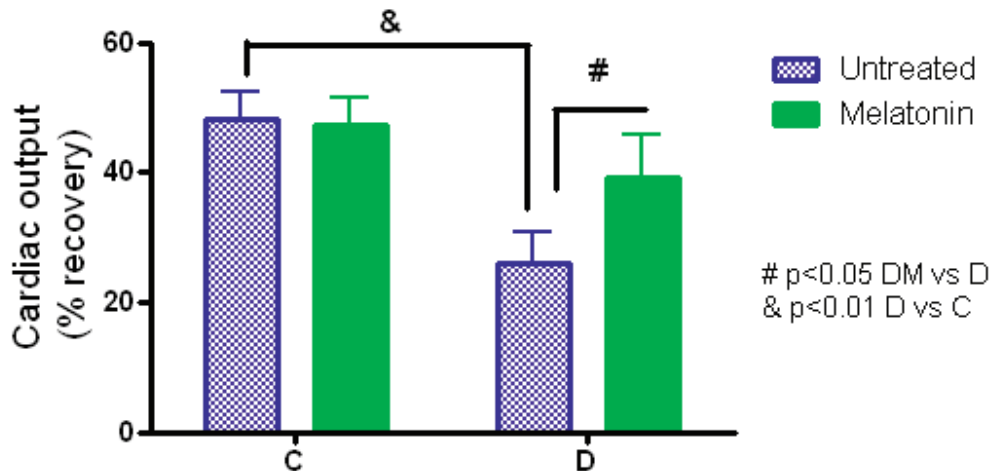


Figure 4- 17 Percentage cardiac output recovery after 60 minutes of reperfusion (n=8-10/group)

Table 4- 3 Summary of pre- and post-iskaemic myocardial function

Time	Parameters	C	CM	D	DM
Stabilization After 30 minutes Before ischaemia (Working heart)	CF	16.1±0.7	13.9±1.3	16±1.2	17.6±0.7
	AO	41.3±2.1	39±3.6	31±1.8 &	37±1.3 #
	CO	57.4±2.3	52.9±4.7	47±3.0	55.3±1.5
	DP	62.6±1.9	62.9±1.8	59±1.4	62.8±2.9
	SP	103±2.6	102±2.6	94±2.8	102±4.8
	HR	235±7.6	205±18	238±6.6	245±10.2
	Wtot	13.6±0.8	11.3±1.3	10±0.9 &	13.4±0.3 #
	dP/dTmax	9.7±0.7	9.8±0.8	8.3±0.9	10.7±0.9
	-dP/dT min	9.24±0.7	10±0.8	7.1±0.6	8±2.2
Reperfusion After 30 minutes Post-iskaemia (Working heart)	CF	11.8±0.7	12.7±1.1	11±1.4	15±1.2 #
	AO	18.9±1.5	18.1±3.4	2.5±2.5 &	9.6±2.5 #
	CO	30.7±2.1	30.8±4	13±3.4&	24.6±2.9 #
	DP	60.8±1.2	64.5±1.8	57±5.0	61.4±1.5
	SP	90.7±1.5	90±1.3	69±5.4	82.8±2.9
	HR	236±8.8	231±15	178±47.5	237±9.8
	Wtot	6.4±0.4	5.7±0.9	1.9±0.72 &	4.5±0.7 #
	dP/dTmax	8.4±0.6	8±0.9	6.5±0.8	7.7±1.0
	-dP/dT min	6.9±0.6	4.5±1.4	5.2±0.2	6.3±0.6
Reperfusion After 60 minutes Post-iskaemia (Working heart)	CF	12.11±0.8	11.5±0.9	10.4±1.1	13.6±0.8 #
	AO	15.2±1.9	10.9±2.5	2.5±2.5 &	7±2.9 #
	CO	27.3±2.0	22.4±2.9	12.8±3.3 &	20.6±3.4 #
	DP	61.8±1.7	62.6±1.6	60.4±4.9	59.7±2.0
	SP	88.8±1.3	88.4±1.6	73.0±4.9	80.6±3.9
	Wtot	5.6±0.4	4.5±0.6	2.59±1.2	4.0±0.8
	dP/dTmax	7.4±0.7	7.7±0.6	4.4±0.5	6.9±1.1
	-dP/dT min	6.8±1.0	7.4±0.5	4.6±0.4	4.8±1.2

p<0.05 (DM vs. D); & p <0.001 (D vs. C)

Table 4- 4. Functional recovery (percentage)

Time	Parameters	C	CM	D	DM
Reperfusion 30 minutes Post-ischaemia	(%) CF	73.8±4.4	92.8±4.2	66.5±5.1	81.2±4.5 #
	(%) AO	46.8±4.6	47.2±7.8	6.2±6.2 &	28.5±6.5 #
	(%) CO	54.03±3.9	60.1±6.2	26.6±5.1 &	44.4±5.2 #
	(%) W tot	48.6±3.9	51.4±5.8	20.4±7.9 &	34.5±5.6 #
	(%) dP/dTmax	91.4±9.4	79.8±3.8	95.3±16.6	75.0±8.3
	(%) -dP/dT min	76.4±5.6	47.4±13.7	88.2±13.6	48.6±12.0
Reperfusion After 60 minutes Post-ischaemia	(%) CF	75.75±5.8	84.5±3.9	64.4±4.4	74.5±3.9
	(%) AO	38.2±5.3	29.3±6.4	6.2±6.2 &	18.7±7.8
	(%) CO	48.3±4.0	47.3±4.2	25.8±4.9 &	37.37±6.2 #
	(%) Wtot	43.4±4.6	39.9±3.5	23.9±8.0 &	30.7±6.2
	(%) dP/dTmax	79.6±9.5	81.7±9.7	39.9±13.6	68.0±10.0
	(%) -dP/dT min	72.7±6.9	77.0±7.0	47.2±14.0	25.0±15.0

Tables 4.3 and 4.4 present the averaged values of pre-and post-ischaemic myocardial function. CF, coronary flow(ml/min); AO, aortic output (ml/min); CO, cardiac output(ml/min); DP, diastolic pressure (mmHg); SP, systolic pressure (mmHg); HR, heart rate (beats /min); Wtot., work total (mWatts), dP/dTmax, contractility index; -dP/dTmin, relaxation index. C, control (n=8); CM control with melatonin (n=9); D, diet (n=8); DM, diet with melatonin (n=10); # p<0.05 (DM vs. D); & p <0.001 (D vs. C)

4.3. MYOCARDIAL INFARCT SIZE

The figure 4.18 represents the effect of diet and chronic melatonin administration on myocardial infarct size. Hearts from the untreated high calorie diet group had an elevated myocardial infarct size compared to the control (38.4± 2.6% (D) vs. 30.4± 1.0% (C), p<0.05). Myocardial infarct size was significantly reduced in treated rats compared to untreated (16.6±2.0% for DM vs 38.4±2.6% for D, p < 0.001), and 12.8±1.5% for CM vs 30.4±1.0% for C, p<0.001). Melatonin reduced infarct size by 57.7% and 58.1% in control and diet groups, respectively.

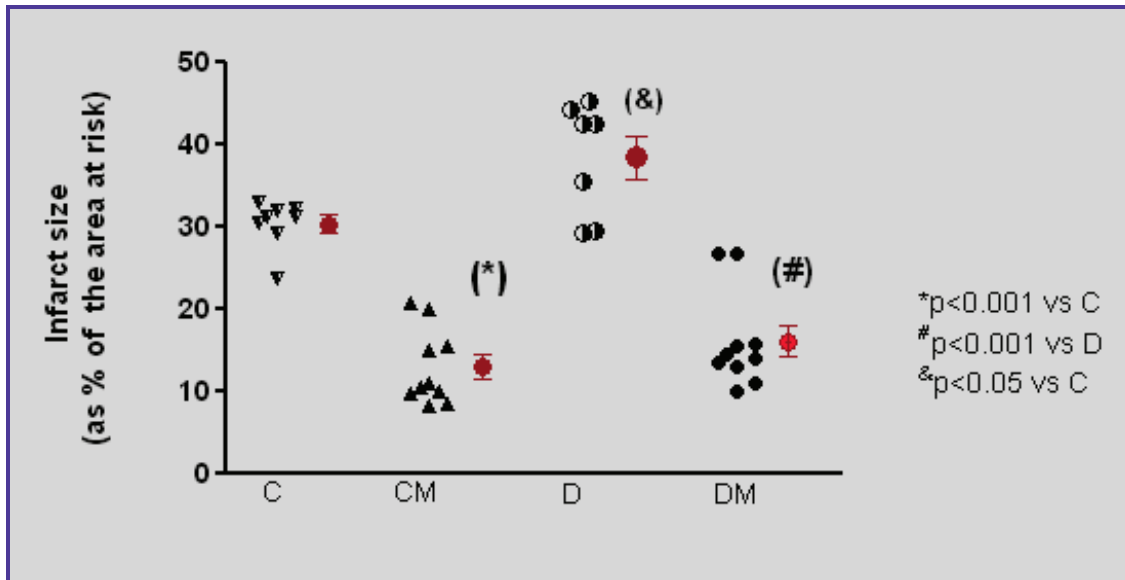


Figure 4- 18 Effect of diet and chronic melatonin administration on myocardial infarct size
C, control (n=8); CM, control with melatonin (n=9); D, diet (n=7); DM, diet with melatonin (n=10).

4.4. CHRONIC MELATONIN AND INTRACELLULAR SIGNALLING

To determine by which signalling mechanism melatonin confers its cardioprotection, we investigated the effect of melatonin on reperfusion injury salvage kinases (RISK) (PKB/Akt and ERKp42/p44), p38 MAPK, AMPK, and GLUT-4 using Western blot analysis. Global ischaemia was chosen to avoid contamination of ischaemic and non-ischaemic tissues which may occur with the regional ischaemia.

4.4.1.Reperfusion injury salvage kinases (RISK) pathway

To determine whether chronic melatonin treatment affects the RISK pathway, baseline and post- ischaemic phosphorylation of PKB/ Akt and ERK 42/44 at 10 minutes reperfusion was assessed.

4.4.1.1. Baseline PKB/Akt and ERK p42/p44

Under baseline conditions, hearts were not perfused. After flushing out of the blood and removing all surrounding tissues, the hearts were freeze-clamped and stored in liquid nitrogen until assayed. Melatonin increased baseline PKB/Akt and ERK 44 activation in control hearts while having no effect in diet groups (fig. 4.19 & 4.20).

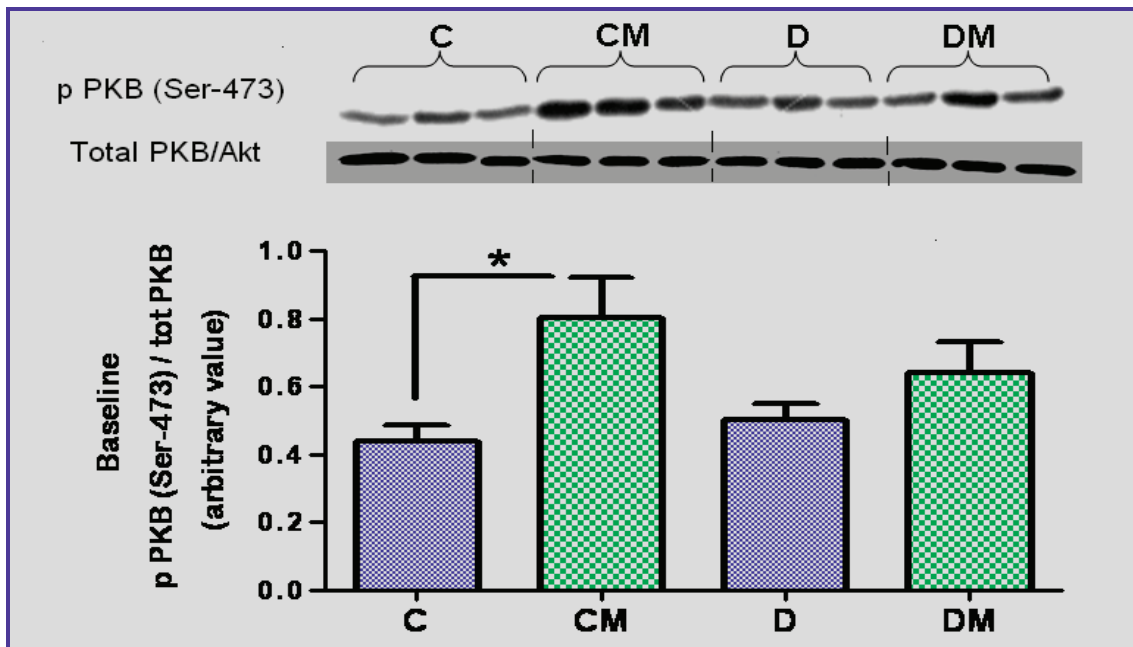


Figure 4- 19 Phosphorylation of PKB/Akt under baseline conditions

* $p < 0.05$ (C vs D) $n = 3$ /group.

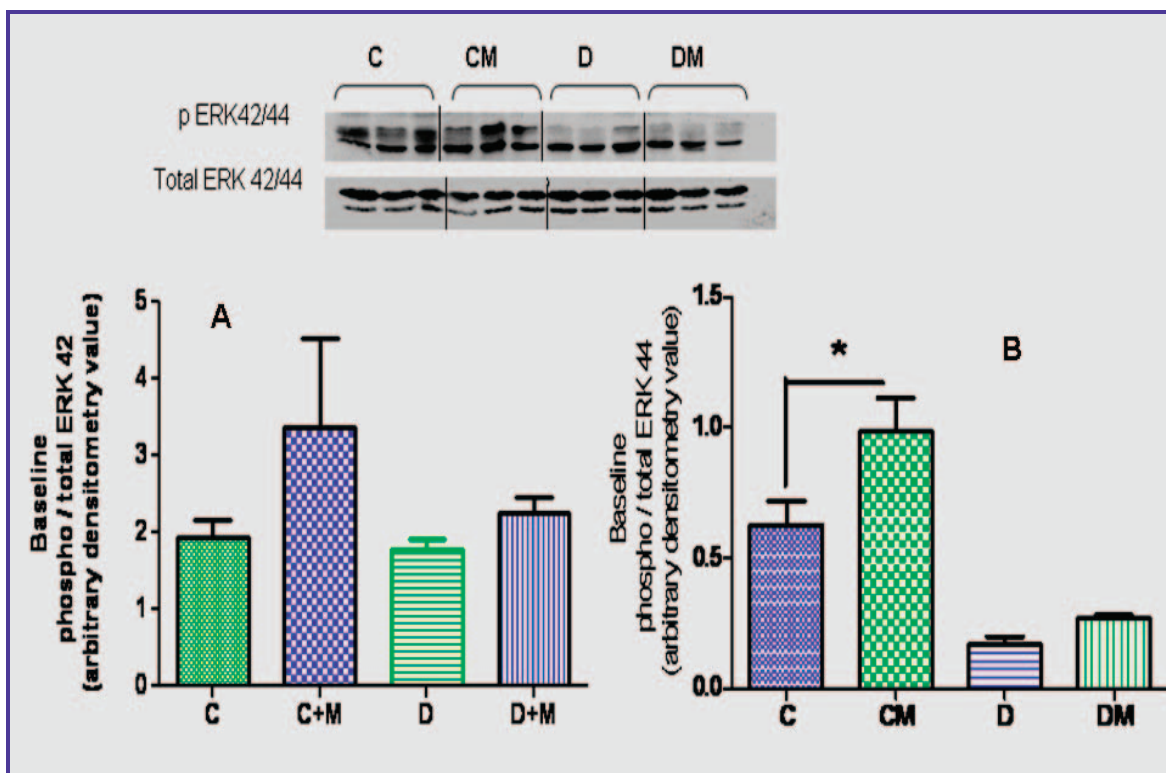


Figure 4- 20 Baseline phosphorylation of ERK p42/p44

Hearts were not subjected to perfusion. (A): ERKp42; (B): ERKp44; * $p < 0.05$ (C vs. CM), $n = 3$ /group.

4.4.1.2. Post-ischaemic PKB/Akt and ERK p42/p44

To evaluate the post-ischaemic protein changes, hearts were freeze-clamped after 10 minutes of reperfusion following global ischaemia. Chronic melatonin treatment increased PKB/Akt activation during reperfusion in the hearts of both control (by 74.2%, $p < 0.05$ C vs. CM) and diet fed animals (by 133.6%, $p < 0.01$, D vs DM) (fig.4. 21). ERK 42 and 44 were only activated by melatonin in the diet fed group while they were not different in control groups (fig.4.22).

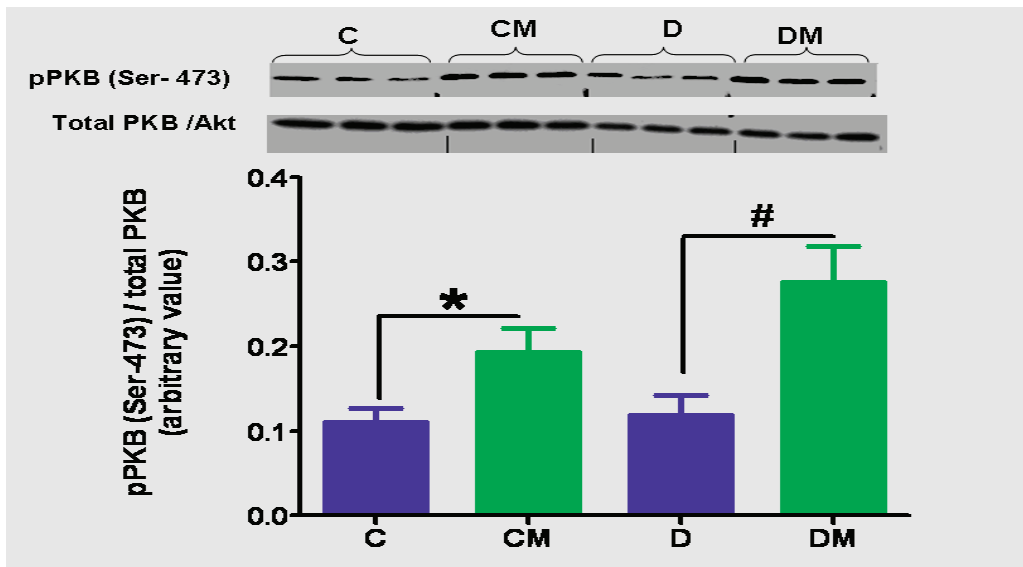


Figure 4- 21 Phosphorylation of PKB /Akt after 10 minutes reperfusion

* $p < 0.05$ (C vs. CM); # $p < 0.01$ (D vs. DM), $n = 6$ /group.

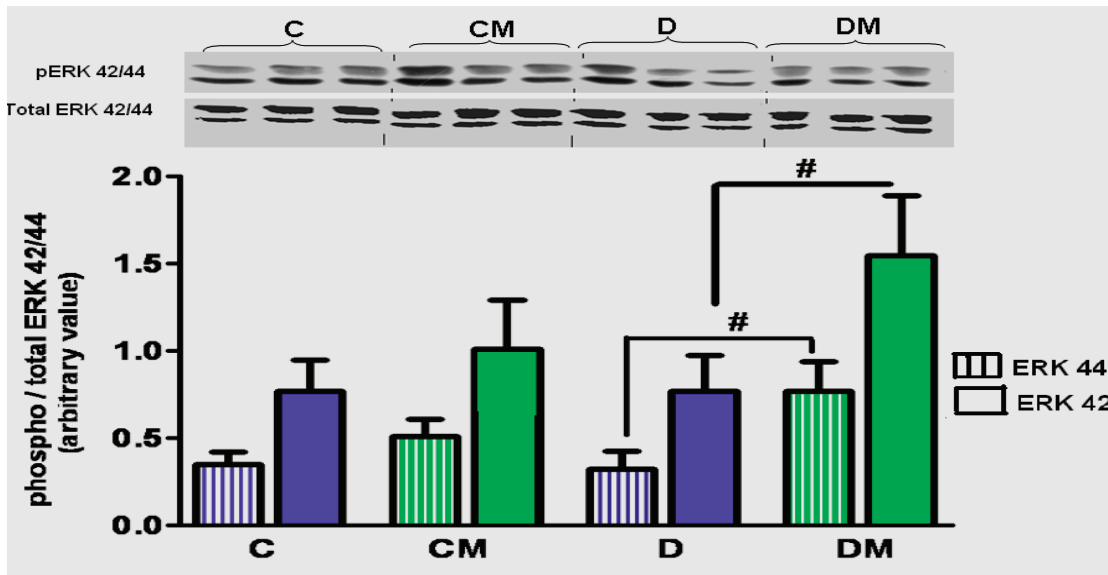


Figure 4- 22 Phosphorylation of ERK 42/44 after 10 minutes reperfusion

$p < 0.05$ (D vs. DM), $n = 6$ /group.

4.4.2. Phosphorylation of p38 MAPK

Chronic melatonin treatment reduced baseline phosphorylation of p38 MAPK in the control group by 77.6 % ($p < 0.05$, C vs. CM) without a significant effect in diet groups (fig. 4. 23). After 10 minutes of reperfusion, melatonin significantly reduced the activation of p38 MAPK in both control (by 31.6 %, $p < 0.05$) and diet fed (by 33. 8 %, $p < 0.05$) groups (fig.4.24).

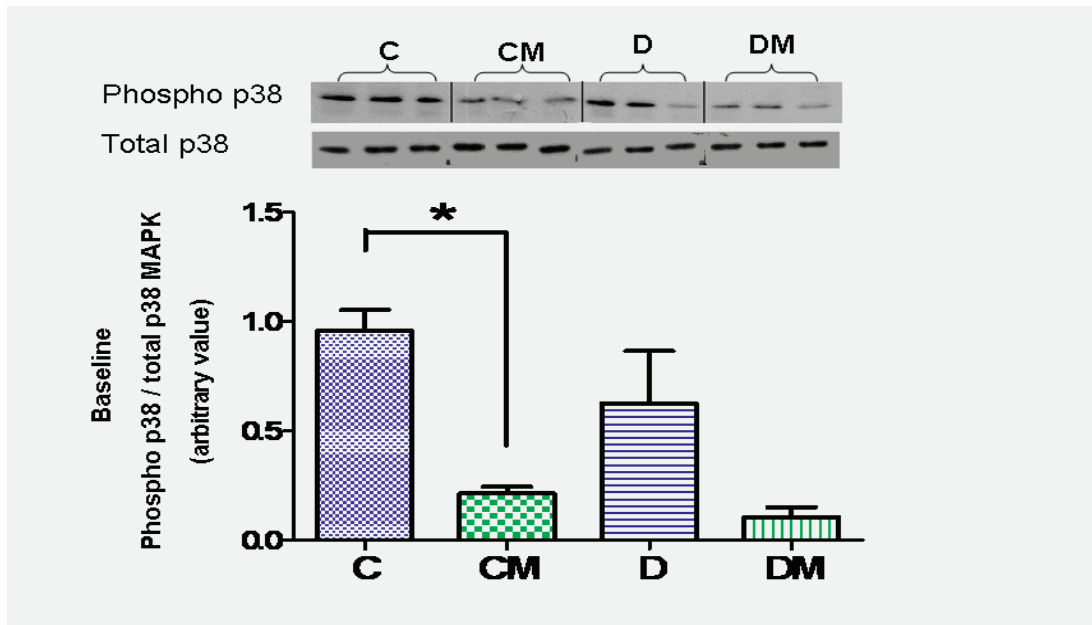


Figure 4- 23 Baseline phosphorylation of p38 MAPK
 $*p < 0.05$ (C vs. CM), $n = 3/\text{group}$

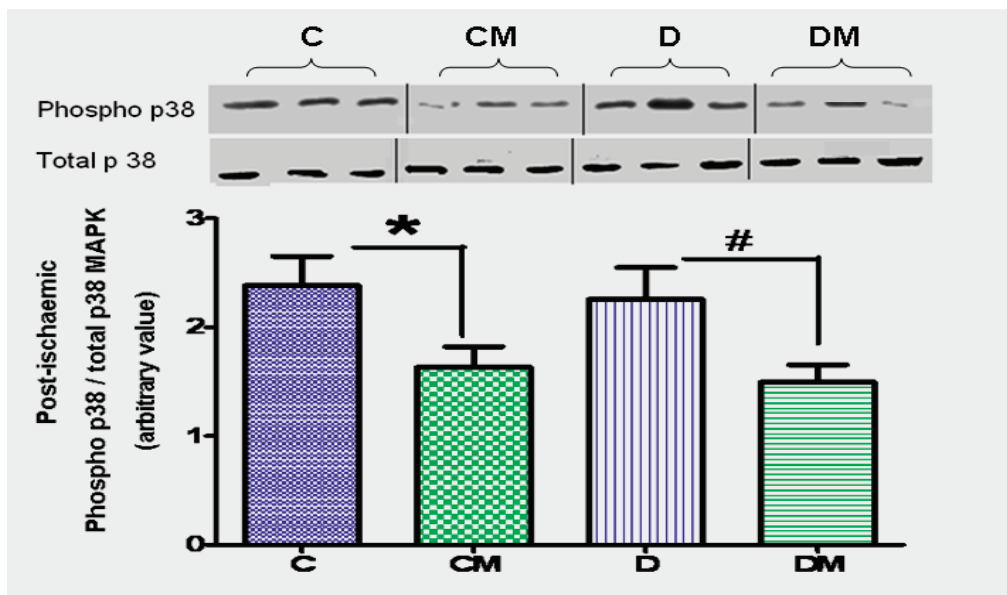


Figure 4- 24 Phosphorylation of p38 MAPK after 10 minutes reperfusion
 $*p < 0.05$ (C vs. CM); $\#p < 0.05$ (D vs. DM), $n = 6/\text{group}$.

4.4.3. AMPK activation and GLUT-4 expression

High calorie diet and chronic melatonin had no effect on AMPK activation and GLUT-4 expression in either control or diet groups (fig. 4.25 & 4. 26).

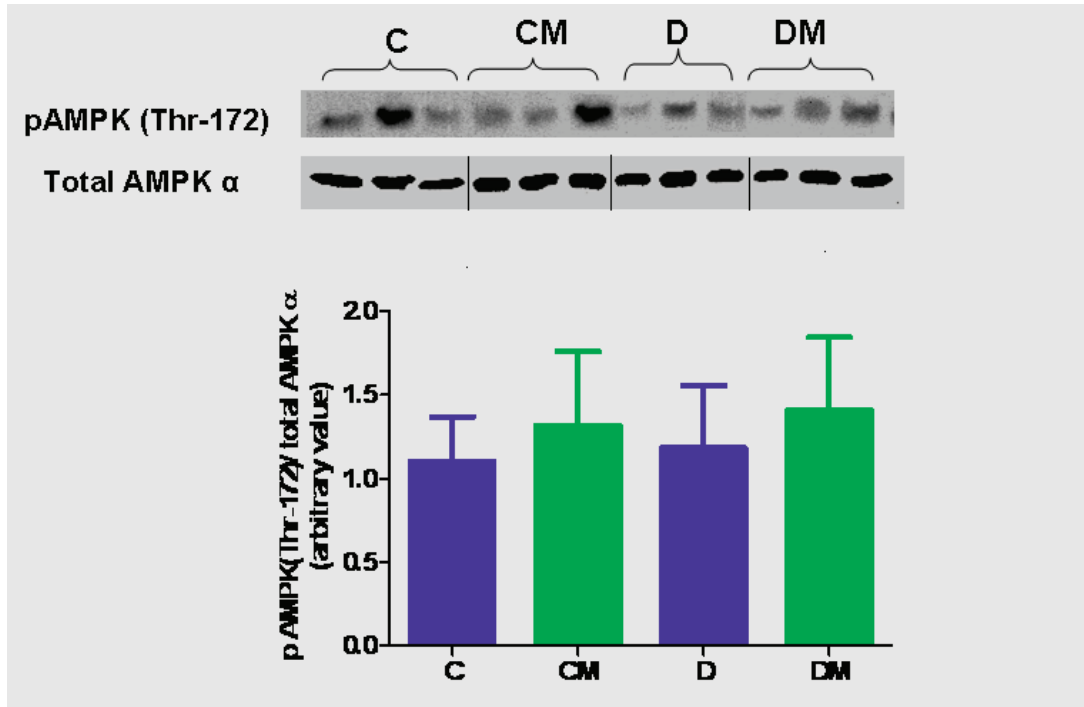


Figure 4- 25 Phosphorylation of AMPK after 10 minutes reperfusion (n=6/group).

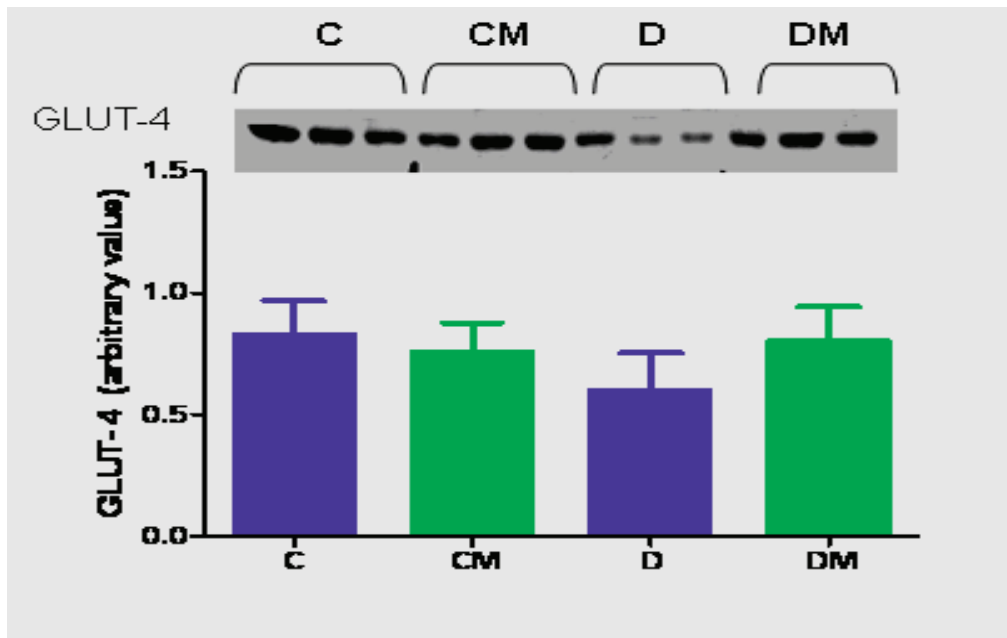


Figure 4- 26 Expression of GLUT-4 after 10 minutes reperfusion (n=6/group).

4.5. CHRONIC MELATONIN CONSUMPTION AND OXIDATIVE STRESS

4.5.1. Chronic melatonin consumption and glutathione levels

To evaluate the myocardial antioxidant status, unperfused hearts were used. Chronic melatonin consumption had no effect on baseline serum and myocardial glutathione content of both control and diet fed groups (fig.4.27 & 28).

Table 4- 5 Glutathione (GSH) levels

<i>PARAMETERS</i>	<i>C</i>	<i>CM</i>	<i>D</i>	<i>DM</i>	<i>n/group</i>
Serum GSH (nmol/mL)	32.5±4.4	39.1±4.6	36.9±0.7	40.6±11.4	6
Myocardial GSH (nmol/g/wet weight)	1.19±0.08	1.18±0.05	1.14±0.05	1.34±0.08	6

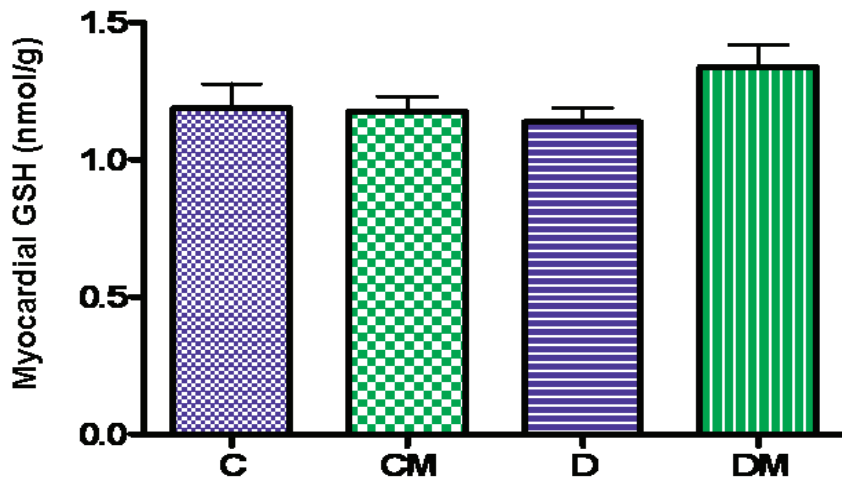


Figure 4- 27 Glutathione (GSH) content in left ventricle (n=6/group)

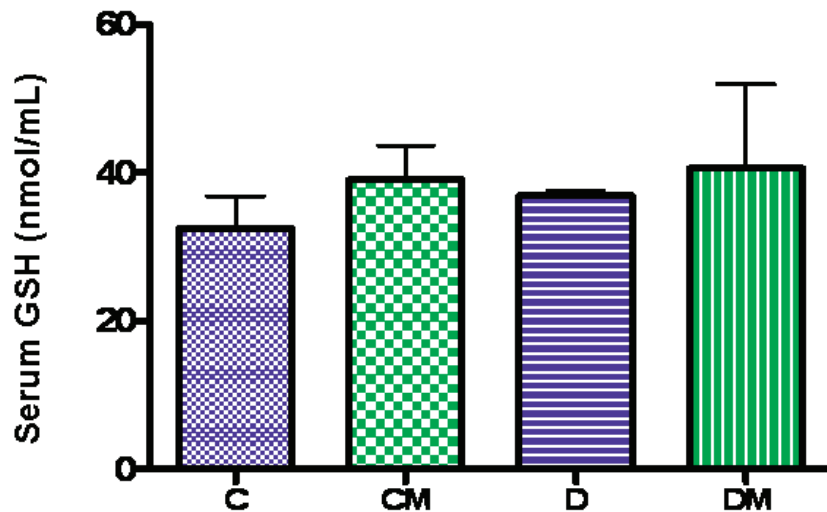


Figure 4- 28 Fasted serum glutathione levels (n=6/group)

4.5.2. Chronic melatonin consumption and lipid peroxidation

To investigate the effects of chronic melatonin consumption on systemic lipid peroxidation, total cholesterol (TC), triacylglycerol (TRIG) phospholipids (PL), conjugated diene (CD), lipid hydroperoxide (LOOH), and thiobarbituric acid reactive substances (TBARS) were measured in sera from non fasted control and diet fed rats with melatonin or drinking water.

High calorie diet did not affect the serum lipid peroxidation compared to control diet (table 4. 6). There were no differences between absolute concentrations of conjugated diene (CD) and thiobarbituric acid reaction substances (TBARS) though there were increases in triglycerides or triacylglycerol (TRIG) and phospholipids (PL) concentrations (table 4.6). This was associated with an increase in body weight gain, confirming the results found on fasting blood lipid profile (fig. 4.1E&D).

In control rats, melatonin treatment has no discernible effect on TRIG, TC or PL in these control rats (table 4. 6). Although the absolute concentration of CD was less with melatonin as a trend, the other two peroxidation products (LOOH and TBARS) were not significantly different. When the lipid peroxidation products were normalised, the difference in CD became significant (fig.4.29), but still no effect was seen on lipid hydroperoxide (LOOH) (fig.4.31) and TBARS (table 4.6).

Table 4- 6 Serum lipid peroxidation

Parameters	C	CM	D	DM
TBARS ($\mu\text{ mol/L}$)	5.46 \pm 1.39	8.71 \pm 2.0	4.77 \pm 0.6	2.42 \pm 0.58(a)
CD ($\mu\text{ mol/L}$)	94.6 \pm 1.74	82.7 \pm 4.14(b)	107.8 \pm 10.88	143.0 \pm 13.82
LOOH ($\mu\text{ mol/L}$)	144 \pm 0.76	144 \pm 1.47	142 \pm 6.5	164 \pm 8.55
TBARS (normalized)	2.38 \pm 0.55	3.31 \pm 0.57	1.39 \pm 0.22	0.64 \pm 0.24(a)
CD (normalized)	41.9 \pm 1.9	33.1 \pm 2.76(c)	30.4 \pm 2.07	33.7 \pm 5.8
LOOH (normalized)	63.8 \pm 3.06	58.6 \pm 6.46	41.3 \pm 4.11	38.5 \pm 5.19
PL (mmol/L)	1.35 \pm 0.06	1.39 \pm 0.09	1.87 \pm 0.25 (d)	2.34 \pm 0.32
TC (mmol/L)	1.76 \pm 0.14	1.61 \pm 0.11	1.56 \pm 0.76	1.75 \pm 0.19
TRIG (mmol/L)	0.93 \pm 0.08	1.21 \pm 0.15	1.84 \pm 0.35(e)	2.41 \pm 0.43
BW (g)	385 \pm 9.7	341 \pm 10.9 (f)	477 \pm 21.6 (e)	436 \pm 12.08

BW, body weight; CD, conjugated diene; LOOH, lipid hydroperoxide; TBARS, thiobarbituric acid reactive substance; TC, total cholesterol; TRIG, triacylglycerol or triglyceride; PL, phospholipid; C, control; CM, control with melatonin; D, high calorie diet; DM, high calorie diet with melatonin. (a), $p < 0.05$ vs. D; (b), $p = 0.65$ vs. C; (c), $p < 0.05$ vs C; (d), $p < 0.05$ vs. C; (e), $p < 0.01$ vs. C; (f), $p < 0.01$ vs. C; $n = 6/\text{group}$.

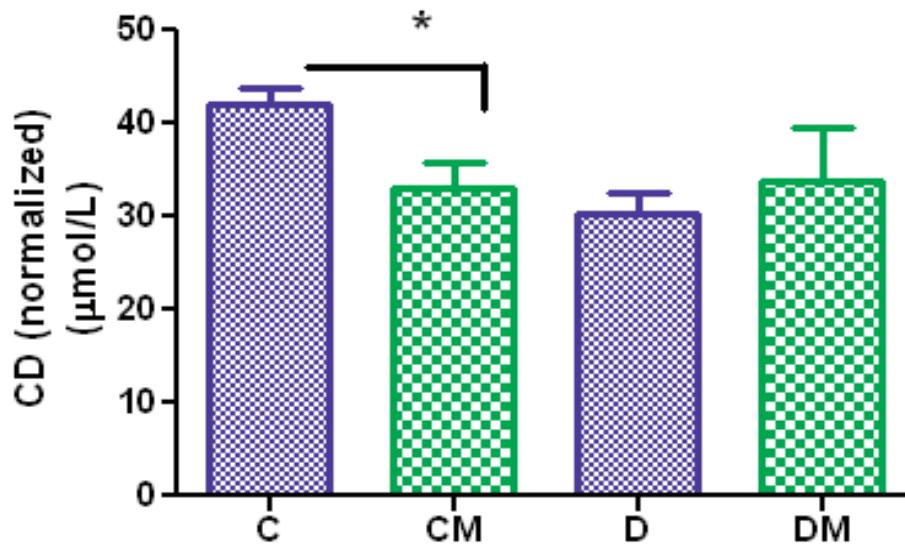


Figure 4- 29 Non fasted serum conjugated dienes (CD)

($n = 6/\text{group}$, $*p < 0.05$ C vs CM)

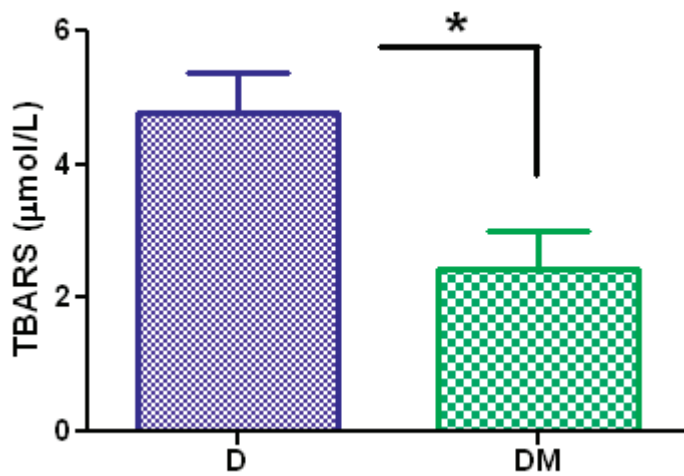


Figure 4- 30 Effect of chronic melatonin on non-fasted serum TBARS (n=6/group)

In diet fed rats, melatonin reduced the absolute TBARS levels by 49.2% ($p < 0.05$, D vs DM) (fig.4. 30.). LOOH and CD levels were not affected (table 4.6 & fig.4. 31). When normalised, only the TBARS were decreased by melatonin in the diet fed rats (table 4.6)

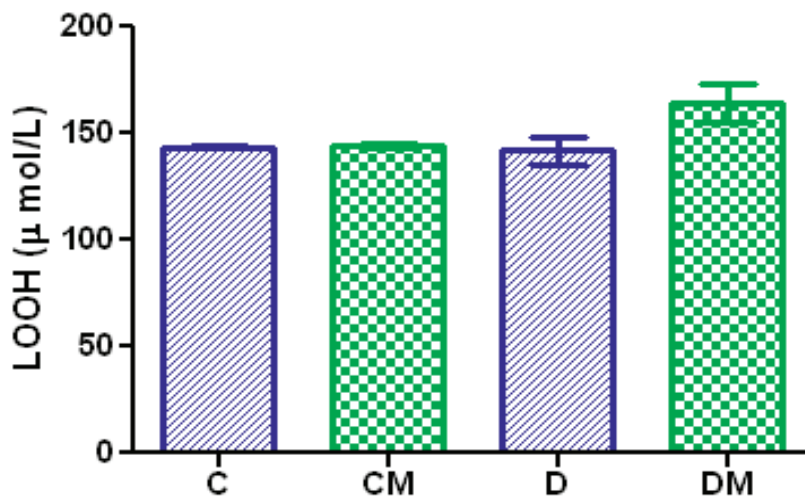


Figure 4- 31 Non-fasted serum lipid hydroperoxide (LOOH) (n=6/group)

CHAPTER FIVE

DISCUSSION

5.1. OVERVIEW OF OUR FINDINGS

The main findings of the present study were as follows. A high calorie diet induced an increase in body weight gain which was characterized by an elevated visceral fat content (fig.4.1A & 4.1B). This weight gain was associated with increased triglyceride, insulin and leptin levels and an increase in the homeostasis model assessment (HOMA) index (table 4. 2; fig.4.1). We also observed a decrease in high density lipoprotein-cholesterol (HDL-C) but no change in glucose levels (table 4.2; fig. 4.8). The diet-induced obesity predisposed the heart to reperfusion myocardial dysfunction and exacerbated infarct size after exposure to ischaemia (fig.4.18). Chronic oral melatonin administration initiated before the establishment of obesity prevented these physiological or metabolic changes associated with increased body weight gain (fig.4.8-13). The chronic administration of melatonin also protected the heart by reducing the myocardial infarct size and improving the post-ischaemic myocardial function (fig.4.18). Melatonin conferred these cardioprotective effects by the activation of the pro-survival kinases (PKB/Akt and ERK42 and 44) with a concomitant inactivation of the pro-apoptotic p38 MAPK during reperfusion (fig.4.21-24). AMPK (5' adenosine monophosphate-activated protein kinase) and GLUT-4 (glucose transporter) expression were not affected by melatonin administration (fig.4.25-26). Melatonin reduced lipid peroxidation as reflected by a reduction in TBARS levels in the high calorie diet fed group (table 4. 6; fig.4.30). Myocardial and serum glutathione levels were not affected by the high calorie diet or melatonin treatment in this study (table 4.5; fig.4.27-28).

5.2. DIET-INDUCED OBESITY

5.2.1. Diet-induced pre-diabetic state

It is well established that a long term positive imbalance between energy intake and expenditure results in obesity (for review see Sethi & Vidal-Puig, 2007). In the present study rats fed a high calorie diet gained more weight than the control rats fed a normal rat chow (fig.4.1A). The high calorie diet fed rats consumed more energy per day than what was

required by their age matched controls. As a consequence of this calories overconsumption, they developed a pre-diabetic state characterized by an increased visceral fat content, elevated circulating insulin and triglycerides levels and reduced HDL-cholesterol levels (table 4.1; fig.4.2). Circulating glucose levels remained unchanged (table 4.2; fig.4.8). Rats fed a high calorie diet developed insulin resistance associated with dyslipidaemia (fig.4.1C, 4.1D & 4.1E). This is in agreement with previous studies in rats (Du Toit et al., 2008; Lima-Leopoldo et al., 2008; Dourmashkin et al., 2005) in mice (Lin et al., 2000; Thakker et al., 2006), and rabbit (Zhao et al., 2008) models of diet-induced obesity. The dyslipidaemia including decreased HDL-cholesterol and elevated triglyceride levels has been described as an early feature of the insulin resistance or metabolic syndrome and frequently precedes glucose intolerance (Reaven, 1988).

Adipose tissue is no longer only considered as energy store, it is now recognized as an endocrine organ that secretes and releases a range of factors that are implicated in carbohydrate and lipid metabolism into the circulation (for review see Kershaw & Flier, 2004 and Vazquez-Vela et al., 2008). In this study, the large visceral fat mass in diet fed rats, implied hypertrophy of adipose tissue which is associated with elevated circulating FFA (Du Toit et al., 2008) and elevated levels of insulin resistance inducing factors such as tumour necrosis factor-alpha (TNF- α) (du Toit et al., 2008) and reduced levels of insulin sensitizing factors such as adiponectin. This would be expected to lead to inhibition of glucose uptake and promotion of free fatty acid oxidation in muscle, which results in a reduced insulin sensitivity contributing to hyperglycaemia. In addition, elevated TNF- α induces the release of free fatty acids (FFA) from adipose tissue into the circulation, promoting hypertriglyceridaemia which is also consistent with the findings of the present study.

The normal glucose levels seen in our model indicate that the animals were in the pre-diabetic state. This refers to the early stage of type 2 diabetes where β -cells secrete sufficient insulin to compensate for insulin resistance and maintain euglycemia (Boden, 2001). Consistent with our study and a previous study done by Du Toit et al. (2008), the pre-diabetic state is very important in the development of cardiovascular disease since pre-diabetic subjects with a high insulin secretory capacity have a higher risk of cardiovascular disease compared with subjects who do not develop insulin resistance (Morel et al., 2005).

5.2.2. Diet-induced resistance to insulin and leptin action

The hormones insulin and leptin are two adiposity signals to the brain (Benoit et al., 2004; Air et al., 2002). Both hormones circulate in direct proportion to the total amount of body fat mass (Rider et al., 2009), both cross the blood-brain barrier by receptor-mediated uptake systems, and both have specific receptors located in regions of the brain associated with the control of body weight (Benoit et al., 2004).

In the present study, the increased visceral fat mass was associated with elevated circulating insulin and leptin levels (fig.4.1B, 1C &1F). This suggests a leptin resistance in our model of diet induced obesity. The association of hyperinsulinaemia with hyperleptinaemia in the present study is in agreement with previous studies in rats (Dourmashkin et al., 2005) and in humans (Ahren et al., 1997; Havel et al., 1996, Rider et al., 2009a).

Leptin resistance refers to a systemic decrease in sensitivity to leptin actions to the extent that normal or increased levels produce an inadequate response (Lin et al., 2000). Amongst other mechanisms (for review see Martin et al., 2008), hyperleptinaemia-induced desensitization of the hypothalamic leptin receptor has been identified as an important cause of leptin resistance (Kim-Motoyama et al., 1997). The primary role of leptin appears to be as a negative feedback adiposity signal that acts in the brain to suppress food intake and increase energy expenditure (Ahima & Osei, 2008). In the common form of obesity, resistance to leptin has been ascribed to diminished transport of leptin across the blood-brain barrier and to elevated hypothalamic levels of suppressor of cytokine signalling 3 (SOCS3) and endoplasmic reticulum (ER) stress, which inhibit leptin signalling (Flier, 1998; Morton et al., 2005; Ozcan et al., 2009). Consistent with this, animals lacking leptin or functional leptin receptors (e.g., leptin resistance) are grossly obese.

The high levels of insulin in our model were also associated with an elevated HOMA index (table 4. 2; fig.4.10) which suggests a reduction in insulin sensitivity in rats fed a diet inducing obesity. Insulin has its primary effects in the periphery where it regulates blood glucose and stimulates glucose uptake by most tissues. In our laboratory cardiomyocytes isolated from the diet induced obesity rats have shown to have a reduced glucose uptake (Huisamen, unpublished data). Analogous to leptin, however, deficits in insulin signalling (e.g., insulin resistance) are associated with hyperphagia in humans. Animals that lack normal insulin signalling in the brain are also obese. Thus, insulin and leptin resistant states reflect the

decrease (or lack) in insulin and leptin sensitivity which has effects similar to those seen with deficiencies of these peptides.

5.3. IMPACT OF THE MELATONIN VEHICLE (0.05% ETHANOL)

Melatonin was dissolved in a small amount of ethanol and added to the drinking water to yield a final concentration of 0.05% (v/v). While epidemiological studies are in agreement about the protective effect of chronic low to moderate alcohol intake, there are conflicting reports regarding the effects of chronic intake of low ethanol concentrations (Dow et al., 2001; Guiraud et al., 2004). Therefore to test the possible long-term additional effects of the vehicle, ethanol (0.05%) was added to the drinking water and its effects documented.

After 16 weeks of administration, ethanol at this dosage did not have any significant effects on body weight, blood glucose and triglycerides as well as HDL-C levels in control or high calorie diet fed rats (fig. 4.2 & 4.3). For example, blood triglyceride levels were (in mmol/L) 0.87 ± 0.08 in controls receiving drinking water only (C), and 0.69 ± 0.12 in controls receiving drinking water with ethanol (CE). In high calorie diet fed rats, triglyceride levels were (in mmol/L) 1.44 ± 0.09 in rats receiving drinking water only (D), 1 ± 0.22 in rats receiving drinking water with ethanol (DE) (fig. 4.3B). It is evident from the above that 0.05% ethanol did not influence our findings. This is in contrast to the findings of Rimm et al. (1999) who demonstrated that light to moderate consumption of ethanol improved lipid homeostasis with increases in HDL-C and reduced platelet aggregation. However, a study done by Dow et al. (2001) using a rat model, found that consuming 15% (v/v) ethanol for 16 weeks failed to protect the heart against ischaemia and reperfusion injury. In addition, it has been shown that 30% (v/v) or less ethanol does not influence diabetes (Vural et al., 2001), the heart, or ion homeostasis (Oz et al., 2006). Guiraud et al. (2004) also reported that chronic intake of 9% ethanol for 7 weeks was the minimal dose to protect the heart without inducing metabolic abnormalities in rats.

5.4. MELATONIN AND DIET-INDUCED SYSTEMIC ALTERATIONS

Melatonin, the primary secretion of the pineal gland, is involved in wide range of physiological actions (for review Pandi-Perumal et al., 2006) and is known to play a role in energy expenditure and body mass regulation in mammals (Bartness et al., 2002). The present study

demonstrated that melatonin is involved in body weight regulation in overweight animals. After 16 weeks of feeding and treatment, rats receiving melatonin in their drinking water had a significantly lower body mass compared to the untreated rats in both control and diet fed rats (fig.4.1). Consistent with our study, daily oral melatonin supplementation has been shown to reduce the body weight of middle-aged rats (Wolden-Hanson et al., 2000). A reduction in body weight gain has also been reported when melatonin was administered intraperitoneally to obese animals (Hussein et al., 2007; Prunet-Marcassus et al., 2003; She et al., 2009).

Although obesity is not accompanied by significant modifications of melatonin secretion in human adults (Rojdmark et al., 1991), the amplitude of the nocturnal pineal melatonin peak decreased significantly in high-fat fed rats (Cono et al., 2008). The role of melatonin in body weight regulation was also evidenced by the observation that the reduction in circulating melatonin in pinealectomized rats was followed by an increase in body weight and administration of melatonin in these pinealectomized animals reduced the body weight gain (Prunet-Marcassus et al., 2003).

The underlying mechanisms by which melatonin affects energy metabolism and prevents body weight gain are not clear. Since melatonin agonist treatment leads to the same effects as melatonin (She et al., 2009), it is suggested that melatonin confers these effects by receptor-mediated pathways. Melatonin's effects are partly mediated through MT₂ receptors present in adipose tissue (Brydon et al., 2001) and Prunet-Marcacuss et al. (2003) have suggested that it could be due to activation of the sympathetic nervous system and its subsequent effects on lipolysis and adipose tissue plasticity (Bartness et al., 2002; Penicaud et al., 2000). When they found no permanent changes in food consumption, Bojkova et al. (2006) proposed that the body mass reduction could be related to improvements in the compromised insulin and leptin signalling associated with obesity. For example, melatonin, leptin and insulin have been found to activate the same intracellular signalling pathways (Carvalho et al., 2001; Morton et al., 2005; Anhe et al., 2004; Picinato et al., 2008). Thus, the interrelationships between melatonin, insulin, leptin and adipose tissue may play a crucial role in weight loss.

In the present study, chronic melatonin treatment reverted insulin and leptin levels of the diet fed animals to control levels (Table 4. 2; fig.4. 9 & 4.11). Adipose tissue weight was also reduced but to a lesser extent than the reduction in insulin and leptin levels (tables 4. 1 & 2; fig.4. 5, 4.9 & 4.11). These data suggest that the reduction in fat content was in part due to

changes in these hormones. Thus, it can be speculated that melatonin may act initially on hypothalamic leptin and insulin receptor sensitivity (as these hormones do under normal conditions) and may consequently relay information about peripheral fat stores to central effectors in the hypothalamus to modify food intake and energy expenditure. On the other hand, *in vitro* melatonin treatment has been shown to possibly inhibit the adipocyte differentiation and limits adipose tissue hypertrophy (Alonso-Vale et al., 2005) by inhibiting fatty acid-induced triglyceride accumulation in cells exposed to physiological levels of oleic acid (Sanchez-Hidalgo et al., 2009). This could explain how melatonin treatment prevented the increase in triglycerides (fig.4.13) and eventually body fat accumulation and body weight gain in the diet-induced obesity group. However, in the control group, melatonin treatment did not affect the lipid profile, insulin and leptin levels (fig. 4.8- 4.13) though the visceral fat and body weight was affected. In non obese rats, the body weight reduction could be linked to the anti-ageing properties of melatonin maintaining the metabolic/physiological parameters at youthful levels (Rasmussen et al., 1999; Wolden-Hanson et al., 2000).

Insulin sensitivity as expressed by the HOMA index was reduced in treated compared with untreated diet fed rats (fig.4.10). Importantly, there was no significant difference between the HOMA index of both control and diet treated rats. Since insulin levels exhibit a nocturnal drop, insulin production has been suggested to be controlled, at least in part, by melatonin. Indeed, both melatonin receptors MT1 and MT2 are expressed in pancreatic islets. Melatonin inhibits insulin secretion in rat pancreatic islets (Picinato et al., 2002) and enhances insulin sensitivity (action) in the central nervous system (Anhê et al., 2004) and peripheral tissues (Sartori et al., 2009). These observations could supply an explanation for the finding that melatonin reduced the fasting insulin levels as well as the HOMA index in the present study and elsewhere (She et al., 2009; Hussein et al., 2007; Wolden-Hanson et al., 2000; Pulschalski et al., 2003).

Our data show that melatonin did not affect blood glucose levels in either control or diet fed rats groups (fig. 4. 8). In this regard, it has to be borne in mind that our model was pre-diabetic exhibiting normoglycaemia. Our study is in agreement with previous studies (Prunet-Marcassus et al., 2003; Wolden-Hanson et al., 2000) and, because there were no melatonin effects on glucose, lipid profile, leptin as well as insulin levels and HOMA index in control rats group (table 4.2 ; fig.4.1- 4.13), melatonin appears to act only when there is a metabolic disturbance. This can explain why, in contrast to the present study, other studies have demonstrated that melatonin improved glucose homeostasis in insulin resistant mice (Sartori et

al., 2009) and reduced circulating glucose levels in the rat (She et al., 2009) and rabbit (Hussein et al., 2007) models of diet-induced obesity.

5.5. MELATONIN AND THE HEART

5.5.1. Melatonin and cardiac remodelling

Cardiac remodelling is strongly associated with obesity (Alpert, 2001; Abel et al., 2008; Chess & Stanley, 2008). The hearts isolated from obese rats weighed more than their age-matched controls (fig.4. 6). However, there was no difference between the heart weight to body weight (HW/BW) ratios of untreated control and diet fed groups (fig.4.7). This is consistent with a previous study (Du Toit et al., 2008) and is possibly due to the fact that the HW/BW ratio may not be an accurate measurement of heart hypertrophy as shown by other authors (Du Toit et al., 2008; Brede et al., 2003; Saupe et al., 2003). In contrast, when the ventricular weight to tibia length (VW/TL) ratio was used as indicator, hearts from obese rats were shown to be hypertrophied (Du Toit et al., 2008). The increased heart weight or cardiac hypertrophy may be the result of the impact of haemodynamic overload and/or increased oxidative stress on the heart in diet-induced obesity (Abel et al., 2008; Chess & Stanley, 2008). Although blood pressure was not determined in the present study to establish a possible link between the blood pressure, body weight and heart hypertrophy, a recent study by Du Toit et al. (2008) reported that 16 weeks of diet-induced obesity was unable to increase the systolic blood pressure in rats. Thus, myocardial hypertrophy could also result from other mechanisms such as insulin resistance and leptin resistance (Rider et al., 2009b). The increases in serum and basal myocardial angiotensin II (AT II) have also been shown to be associated with hypertrophy in diet-induced obesity (Du Toit et al., 2005).

Chronic melatonin treatment reduced the HW (fig.4.6) and consequently the HW/BW ratio in all groups (fig.4.7). The reduction in HW was associated with a reduction in body weight and fat mass and improvement in leptin and insulin sensitivity (reduction of HOMA index) in diet fed rats as discussed above. The mechanisms of HW reduction by melatonin are not clear. Beside the possible reduction in haemodynamic overload *via* the antihypertensive effects of melatonin (Paulis & Simko, 2007), the reduction in visceral fat may also be involved (Rider et al., 2009a). In our study, the reduction of HW (21% in control and 29.2% in diet) did not follow the same pattern as did BW reduction (10.7% in control and 8.1% in diet). Therefore, in diet fed rats, the

reduction in HW/BW ratio could be due to other factors such as increased insulin or leptin sensitivity. Rider et al. (2009a) have recently suggested that the cardiac hypertrophy regression, as consequence of weight loss in obesity, is the result of the increase in expression of leptin receptors in the myocardium. This could possibly explain how melatonin prevented the increase in cardiac hypertrophy in diet-induced obesity rats. However, in control rats, the mechanism remains unclear. After 10 weeks of melatonin administration, Markova et al. (2003) have also reported a reduced heart weight in young male rats fed a normal rat chow. Melatonin was recently shown to have antifibrotic effects in hypertensive rats (Paulis et al., 2009) as well as antihypertrophic effects in hyperthyroid rats (Ghosh et al., 2007). This may be due to the direct and indirect antioxidant properties of melatonin (Simko et al., 2009). Whether there is a direct effect of melatonin on the heart weight and how it is accomplished requires additional investigation.

5.5.2. Melatonin and myocardial function

This section focuses on the basal function of the heart during normoxic conditions, before induction of ischaemia (the effects on post-ischaemic function will be addressed in the next section). It is well established that obesity is associated with myocardial dysfunction (Poirier et al., 2006) and in the present study, isolated hearts from high calorie diet fed rats exhibited decreased basal myocardial function (aortic output and work total) as measured *in vitro* (table 4. 3). This is in agreement with previous *in vitro* studies (Akki & Seymour, 2009; Essop et al., 2009; Du Toit et al., 2005; Du Toit et al., 2008). The poor myocardial function in obesity has been ascribed to metabolic abnormalities including dyslipidaemia, glucose intolerance, insulin and leptin resistance, associated with impaired myocardial Ca^{2+} handling (Banerjee & Peterson, 2007; Lopaschuk et al., 2007; Chess & Stanley, 2008). Obesity induced by a high-fat diet causes cardiac upregulation of Ca^{2+} transport-related genes in the sarcoplasmic reticulum (Lima-leopoldo et al., 2008). This is also associated by mitochondrial dysfunction characterized by inefficient nutrient oxidation causing low ADP/O ratios (Kim et al., 2008; Essop et al., 2009). Mitochondrial dysfunction also leads to an increased production of reactive oxygen species subsequent to inflammation, which exacerbates the insulin resistant state and causes poor cardiac contractility (Kim et al., 2008; Bugger & Abel, 2008).

Chronic melatonin treatment had no significant effects on myocardial function in controls as measured *in vitro* (table 4. 3). This is consistent with previous studies where melatonin administration had no significant effect on hemodynamic parameters before ischaemia *in vivo* (Sahna et al., 2005; Lee et al., 2002) and *in vitro* (Szarszoi et al., 2001; Lochner et al., 2006). However, chronic melatonin treatment improved the basal myocardial function (aortic output and work total) of diet fed group (table 4.3). This could be due to the prevention of age-dependent cardiac mitochondrial dysfunction (Reiter et al., 2002; Rodriguez et al., 2007) and improvement of myocardial calcium homeostasis by melatonin (Salie et al., 2001; Yeung et al., 2008).

5.5.3. Melatonin and ischaemia reperfusion injury

Ischaemia/reperfusion damage is characterized by myocardial dysfunction, arrhythmias and cell death or myocardial infarction (for review see Opie, 2004). It is the consequence of metabolic alterations resulting from insufficient oxygen supply or the hypoxic state during sustained ischaemia. This is followed (at reperfusion) by excessive oxygen/nitrogen radical production and increased Ca^{2+} overload, associated with the altered myocardial metabolism. In addition reperfusion is associated with restoration of physiological pH, inflammation and the opening of the mitochondrial permeability transition pore (MPTP) (non selective pore) (for review see Buja et al., 2005; Yellow & Hausenloy, 2007).

5.5.3.1. Diet-induced obesity and ischaemia/reperfusion injury

Epidemiological and animal studies have demonstrated that obesity and its related metabolic abnormalities including insulin resistance and dyslipidaemia have been associated with increased incidence of myocardial infarction (Fedorowski et al., 2009; Prasad et al., 2009; Ranjith et al., 2007; Porier et al., 2006). In the present study, after coronary artery occlusion and 30 minutes of reperfusion, isolated hearts from high calorie fed rats had a decline in post-ischaemic myocardial function (aortic output and work total) compared to control rats (table 4.3 & 4.4). In addition, the hearts from the diet fed group had an increased infarct size (by 26 %) compared to their age matched controls (fig.4.18). This indicates an increased susceptibility to ischaemia/reperfusion damage for the diet-induced obesity rat hearts, consistent with several

previous studies (Akki & Seymour, 2009; Essop et al., 2009; Du Toit et al., 2005; Du Toit et al., 2008; Morel et al., 2003).

The mechanism linking obesity and myocardial I/R injury are poorly understood. Metabolic alterations associated with increased cytosolic Ca^{2+} accumulation, increased reactive oxygen species and mitochondrial dysfunction may play an important role (Kim et al., 2008). Thus, the exacerbation of post-ischaemic function recovery in obese animal could be attributed to a number of mechanisms, including increased ceramide production, lipoapoptosis, oxidative and nitrosative stress, inefficient energy metabolism, and alterations in cardiomyocyte Ca^{2+} regulation (Banerjee & Peterson, 2007; Chess & Stanley, 2008). The I/R injury in diet-induced obesity has been associated with increased cardiac inflammatory markers including chemokines and cytokines (Thakker et al., 2006), elevated serum TNF- α (Du Toit et al., 2005) and high circulating free fatty acids (Thakker et al., 2008). It could also be the consequence of cascades of maladaptative metabolic changes of *in vivo* FFA metabolism (Morel et al., 2003; Lopaschuk et al., 2007).

However, reports on ischaemia/reperfusion injury in animal models of obesity have been controversial: some investigators have found increased myocardial susceptibility to ischaemia and reperfusion injury consistent with our findings (as stated above), while other could not find any difference (Thim et al., 2006). Surprisingly, others have found a resistance to ischaemia and reperfusion injury with obesity (Bouhidel et al., 2008; Kristiansen et al., 2004). These inconsistencies have been attributed to differences in body weight gain, type and age of animal model (Wang et al., 2004; Mozaffari et al., 2008). It has been noted that mild diabetes generally renders the heart resistant to ischaemic injury since glucose treatment protected cardiomyocytes with diabetic phenotype against necrosis (Mozaffari et al., 2008).

5.5.3.2. Effect of melatonin on functional recovery and infarct size

In the present study, chronic melatonin did not affect the post-ischaemic myocardial function in control rats (tables 4.3 & 4.4; fig.4.14-17). However, infarct size was reduced by 58.1% (fig.4.18). This is consistent with previous studies on acute administration of melatonin before coronary artery ligation *in vivo* (Sahna et al., 2005; Lee et al., 2002) or *in vitro* (Lochner et al., 2006) showing a reduction in infarct size with no significant effect on hemodynamic parameters during ischaemia and during reperfusion. The mechanism by which chronic melatonin

treatment improves ischaemia and reperfusion injury remains under investigation. Interestingly, in the isolated hearts from non obese rats, the cardioprotective effects of oral melatonin treatment persist for almost 4 days after melatonin withdrawal (Lochner et al., 2006). Therefore, the observed cardioprotection could result from long term effects of melatonin *via* stimulation of antioxidant enzymes (Rodriguez et al., 2004). In this regard, it can be speculated that chronic melatonin increased the antioxidant capacity of the heart by upregulation of antioxidant enzymes, changes in receptor populations, and their signalling (Lochner & Genade, Unpublished observations) which protect against the increase in intracellular calcium overload and free radical generation, the principal features of I/R injury (Yellon & Hausenloy, 2007). Melatonin has been shown to reduce infarct size by increasing the antioxidant glutathione (GSH) *in vivo* (Sahna et al., 2005) and reducing ROS generation and lipid peroxide concentration *in vitro* (Kaneko et al., 2000) in the heart.

Beside the long term melatonin administration, using acute melatonin treatment, previous convincing *in vitro* and *in vivo* studies have demonstrated the protective effects of melatonin against I/R injury (Reiter & Tan 2003; Paulis & Simiko, 2007; Tengattini et al., 2008; Kucukakim et al., 2009). In addition to the heart, melatonin also reduced the molecular and cellular damage resulting from I/R in other organs (Reiter et al., 2005; Gurlek et al., 2006; Siu et al., 2006). This protection was attributed to its free radical scavenging and broad-spectrum antioxidant properties. On the other hand, acute melatonin administration was recently shown to protect the isolated heart *via* its anti-adrenergic actions (Genade et al., 2008). The anti-adrenergic effects of melatonin have also been demonstrated in rat (Abete et al., 1997) and guinea pig (Arvola et al., 2006) heart papillary muscle.

The reduction of infarct size was not accompanied by an improvement of myocardial function in control rats (CM) (in contrast to diet fed rats) (tables 4. 3 & 4; fig.4.14-17). This has been also reported by previous studies on acute melatonin effects *in vivo* (Lee et al., 2002 and Sahna et al., 2005) and *in vitro* (Lochner et al., 2006 and Genade et al., 2008). A previous study done by Lochner et al., (2003) has demonstrated that a reduction in infarct size is not always accompanied by an improvement in functional recovery during reperfusion, due to concomitant stunning. In contrast, the oral melatonin pre-treatment (40 µl/mL) for 7 days followed by 24 hours of withdrawal, reduced infarct size and improved post-ischaemic function of the hearts subjected to 35 minutes coronary artery occlusion *in vitro* (Lochner et al., 2006). In our study, although melatonin was also dissolved in ethanol and added to the drinking water

at the same amount, it was orally administered for 16 weeks instead of 7 days (Lochner et al., 2006), without any withdrawal of melatonin before the heart perfusion. Moreover, in the same study by Lochner et al. (2006), the short-term oral melatonin at the dose of 20µl/ml failed to protect the heart. It is therefore evident that the effects of melatonin depend on the dose administered, the time and the way of administration as well as the protocol followed as it was also noted by other authors (Szarszoi et al., 2001; Chen et al., 2003; Arvola et al., 2006). The isolated hearts from diet fed rats treated with melatonin had improved post-ischaemic myocardial function (aortic output and work total) (table 4.4). This may suggest that the beneficial effects of melatonin in DIO override the effect of stunning during reperfusion.

This is the first study investigating the effects of melatonin on the heart in obesity. Several factors eliciting protection should be considered: this could be related to the prevention of insulin and leptin resistance seen in diet fed rats and the associated metabolic alterations. This prevention is possibly associated with a decrease in reactive oxygen species generation and intracellular Ca^{2+} accumulation (see myocardial function). Interestingly, in diet fed rats, melatonin reduced systemic lipid peroxidation which could also play a role in the observed cardioprotection (Bertuglia & Reiter (2007). The other features of cardioprotection are discussed in the following section.

5.5.4. Melatonin and cardiac intracellular signalling

5.5.4.1. Reperfusion injury salvage kinases pathway

To determine the effects of chronic melatonin administration on intracellular signalling in the normoxic heart under baseline conditions, hearts were freeze-clamped immediately after removal from the body and stored in liquid nitrogen until assayed for kinases of the RISK pathway (PKB/Akt, ERK42 and 44) and the pro-apoptotic kinase p38 MAPK. To evaluate the post-ischaemic protein changes, a model of global ischaemia was used to obtain a more homogeneous sample.

Under baseline conditions, isolated hearts from rats fed a high calorie diet did not show any differences in the activation of PKB/Akt (fig.4.19), ERK42 (fig.4.20 A), and p38 MAPK (fig.4.23) compared to their age-matched controls. ERK 44 activation was reduced in diet fed rats (fig.4.20B). In contrast, Li et al. (2005) have reported a significant increase in p38 MAPK activation in the hearts from obese diabetic rats. This discrepancy is probably due to the fact

that we employed a pre-diabetic model. However, chronic melatonin treatment increased the activation of PKB/Akt and ERK 44 in control rats (fig.4.19 & 4.20) associated with a concomitant reduction in activation of p38 MAPK (fig.4. 23). Interestingly melatonin treatment had no effect on the basal activation patterns of these kinases in hearts from diet fed animals, which may be due to harmful effects of obesity. However, the reasons for these observations should be further investigated.

To evaluate the long term effects of melatonin treatment on post-ischaemic protein changes, hearts were freeze-clamped after 10 minutes reperfusion following 20 minutes global ischaemia. The high calorie diet did not affect the post ischaemic activation of PKB/Akt, (fig.4.21), ERK 42 and 44 (fig.4.22) and p38 MAPK (fig.4.23) compared to the control groups. In the control rats, melatonin increased post-ischaemic PKB/Akt but not ERK 42/44 and reduced p38 MAPK activation during reperfusion. In the diet fed rats, melatonin increased post-ischaemic PKB/Akt and ERK 42/44 and reduced p38 MAPK activation (fig.4.21, 4.22 & 4. 24). These findings are consistent with previous studies where it has been shown that acute melatonin treatment of isolated rat hearts during reperfusion activated the RISK pathway and reduced p38 MAPK (Genade et al., 2008). In addition, after administration of melatonin in rats, Anhê et al. (2004) also reported *in vivo* activation of PKB/Akt and ERK42 and 44 in the hypothalamus. It is well-established that activation of the reperfusion injury salvage kinase pathway (RISK) is associated with cardioprotection (Hausenloy and Yellon 2007). Our results suggest that melatonin also induces cardioprotection *via* these kinases. A link has been proposed between the RISK pathway and the mitochondrial permeability transition pore and recently, convincing evidence has been presented that the cardioprotective effects of melatonin are due to inhibition of the mitochondrial permeability transition pore (MPTP) opening (Petrosillo et al., 2009). In addition to activation of the RISK pathway, it has recently been shown that acute administration of melatonin to the isolated rat heart caused activation of STAT-3 (Lecour S, Unpublished observations), which could also act on the mitochondrial membrane permeability transition pore.

Finally, the exact mechanism whereby melatonin increased activation of PKB/Akt and ERK42/44 and reduced p38 MAPK activation in diet fed rats remains unknown. This could merely be a reflection of protection caused by the other effects of melatonin or due to effects of melatonin on the activity of phosphatases. The latter possibility has not yet been examined. As discussed above, high calorie diet induced-obesity is associated with systemic metabolic

alterations (increased circulating insulin and leptin) compared to the control rats. It has been shown that crosstalk occurs between insulin and leptin signalling, both activating the insulin receptor substrate (IRS)-phosphatidylinositol-3-OH kinase (PI3K) pathway (Morton et al., 2005) and the STAT-3 pathway (Carvalho et al., 2001). It can therefore be speculated that melatonin reverses the insulin resistance by mimicking the actions of insulin and leptin signalling *via* cross-talk between these signalling pathways, all leading to PKB/Akt and STAT-3 activation. However, this possibility needs further investigation.

5.5.4.2. AMPK and GLUT- 4

Since we found that melatonin is implicated in body weight regulation, which is one of the features of metabolism abnormalities, we decided to examine the effects of melatonin on the activity of proteins involved in cardiac metabolism. In this regard we examined the effect of melatonin on post-ischaemic AMPK activation. AMPK is considered a cellular energy metabolism sensor (for review see Hardie, 2004; Young et al., 2005). However, chronic melatonin administration did not affect AMPK activity (fig.4.25), a finding consistent with previous studies. The administration of melatonin to C₂C₁₂ murine skeletal muscle (Hâ et al., 2006) and hepatic (Shieh et al., 2009) cells did not affect the activity of AMPK-mediated pathway.

In addition, we investigated the expression of the glucose transporter (GLUT-4), to determine whether melatonin possibly influenced carbohydrate metabolism in the heart. However, chronic melatonin had no significant effect on total GLUT-4 expression (fig.4.25). Sartori et al. (2009) did not find an effect of melatonin administration (i.p.) on *in vivo* skeletal muscle glucose uptake of obese mice. In contrast, previous studies using hepatic (Shieh et al., 2009) and skeletal muscle (Hâ et al., 2006) cells have demonstrated that melatonin treatment did increase glucose uptake. This possibly occurs by activation of PKC- ζ and not AMPK-mediated pathways (Hâ et al., 2006). This possibility should be further evaluated in our model.

5.6. MELATONIN AND OXIDATIVE STRESS

Oxidative stress has been implicated in the aetiology and exacerbation of cardiovascular disease (Dhalla et al., 2000b). To investigate the myocardial antioxidant status in our model and the effects of melatonin, the myocardial (unperfused hearts) and serum glutathione levels

were determined in control and diet fed rats. Overnight fasted animals were used for these experiments. In addition, serums from non fasted animals were assayed to quantify lipid peroxidation, a feature of oxidative damage.

5.6.1. Melatonin and glutathione (GSH)

Glutathione or L- γ -glutamyl-L-cysteinyl-glycine, or GSH, is the major low-molecular-mass thiol compound in plants and animals which lacks the toxicity associated with cysteine.

It is therefore considered as a cellular thiol “redox buffer” to maintain a given thiol/disulfide redox potential and is regarded as the key antioxidant in animal tissue (Akerboom & Sies, 1981).

In the present study, melatonin treatment had no effect on myocardial or serum glutathione in both control and diet fed rats (table 4.5; fig.4. 27 & 4. 28). In addition, serum and myocardial glutathione levels of the high calorie diet group were similar to those of control group. Consistent with our study, Essop et al. (2009) have also found no difference in the degree of oxidative stress-induced damage between rats fed a high calorie diet (for 16 weeks) and their age-matched controls. In the study done by Vincent et al. (1999) diet-induced obesity predisposed the myocardium to oxidative stress (lipid peroxidation) but the antioxidant glutathione levels were not different between obese and lean rats.

Our data suggest that the pre-diabetic state does not affect cardiac glutathione content, consistent with the previous study by Vincent et al. (1999). In addition, melatonin treatment was without effect on this parameter. These findings are surprising since melatonin can activate glutathione reductase (GRd), glutathione peroxidase (GPx) and glucose-6 phosphate dehydrogenase (Reiter & Tan, 2003; Tengattini et al., 2008) and elevated levels of glutathione have been associated with the cardioprotective effects of melatonin treatment *in vivo* (Sahna et al., 2005).

5.6.2. Melatonin and lipid peroxidation

Accumulation of excessive FFA as triglyceride droplets in muscle and other non adipose tissues can induce excessive reactive oxygen species production and increase the level of oxidative stress which is implicated in pathogenesis of insulin resistance (Grattagliano et al., 2008) and obesity (Vincent et al., 2007). The high calorie diet did not affect the serum lipid

peroxidation compared to control (table 4. 6). There were no differences between absolute concentrations of conjugate diene (CD) and thiobarbituric acid reactive substances (TBARS) though there were increases in triglyceride (TRIG) and phospholipid (PL) concentrations. This was associated with an increase in body weight gain, confirming the results found on fasting blood lipid profile (table 4. 2). It increased TRIG and PL, presumably through increased VLDL containing fatty acids from condensed milk (saturated fatty acids) and/or endogenous synthesis (of saturated or monounsaturated fatty acids) (She et al., 2009).

In the control rats, melatonin treatment had no discernible effect on TRIG, TC or PL (table 4. 6). Although the absolute concentration of CD tended to be less with melatonin, the other two peroxidation products (LOOH and TBARS) were not significantly different. When the lipid peroxidation products were normalised, the difference in CD became significant (see fig 4.29) but still no effect was seen on lipid hydroperoxide (LOOH) and TBARS. This may be indicative of the antioxidant activity of melatonin, or due to an effect of melatonin on unsaturated fatty acid metabolism (Nishida et al., 2002).

TBARS assay reflects the overall oxidative stress (Anurag & Anuradha, 2002). In diet fed rats, melatonin treatment did not affect LOOH and CD levels (fig. 4. 29 & 31) while the absolute and normalized TBARS levels were reduced (table 4.6; fig.4. 30). Since this does not appear to be the case in the control rats, it may be related to an effect of high calorie diet, where lipid peroxidation could be enhanced by the weight gain itself (decreased by melatonin) or the lipid metabolism relating to the liver and/or adipose tissue (Bonet et al., 2007). Although we did not measure lipid peroxidation in fasted serum to make a correlation, insulin levels and HOMA index have been identified as strong determinants of TBARS levels, suggesting a role for insulin resistance in increased lipid peroxidation or oxidative stress (Anurag & Anuradha, 2002).

Consistent with our findings melatonin was shown to reduce plasma lipid peroxidation (TBARS levels) in cardiomyopathic hamsters (Bertuglia & Reiter, 2007). In addition, melatonin reduced lipid peroxidation in post-ischaemic myocardial tissue and protected against apoptosis (Dobsak et al., 2003). These antioxidant properties of melatonin have been repeatedly demonstrated by several workers (Reiter & Tan, 2003, Tengattini et al., 2008) and are achieved *via* its direct free radical scavenging and antioxidant enzyme stimulatory activities.

CHAPTER SIX

CONCLUSIONS AND FUTURE PERSPECTIVES

6.1. CONCLUSIONS

The strong association between obesity and cardiovascular disease has prompted several researchers to investigate their complex interrelationships. To our knowledge, this is the first study to investigate the effects of melatonin on the heart in obesity and insulin resistance. Consistent with previous studies, the present study demonstrated successfully how diet-induced obesity increases myocardial susceptibility to ischaemia and reperfusion injury. The present study also demonstrated clearly the beneficial role of chronic melatonin consumption on myocardial function and ischaemia/reperfusion injury in a rat model of diet-induced obesity.

Chronic oral melatonin treatment reversed the metabolic profile characteristic of dyslipidaemia, insulin resistance, and leptin resistance associated with increased body weight. It maintained glucose concentration at control levels and protected the heart against ischaemia/reperfusion induced damage. Chronic melatonin treatment activated the reperfusion injury salvage kinase (RISK) pathway and reduced the pro-apoptotic kinase p38MAPK activation during reperfusion. Myocardial AMPK activation and GLUT-4 expression were however not affected by the treatment. In addition to its signalling effects, melatonin treatment reduced the lipid peroxidation (TBARS levels) in diet fed rats while having no effect on lipid peroxidation in control rats. Myocardial and serum glutathione were also not affected by diet or/and melatonin treatment.

Our original hypothesis was that melatonin, in view of its anti-oxidant and free radical scavenging properties could abrogate the harmful effects of obesity. In view of the development of a pre-diabetic state only, it is possible that free radicals did not play such an important role in this model of diet-induced obesity. However melatonin pretreatment did reduce lipid peroxidation in the obese animals, suggesting some role for its anti-oxidant and free radical scavenging properties in this scenario. In view of previous results from our laboratory (Lochner et al., Unpublished data) which showed that melatonin receptor antagonism abolished its long-term beneficial actions, as well as the findings of the present study, we would like to suggest that melatonin's beneficial actions are very definitely receptor-mediated to a large extent.

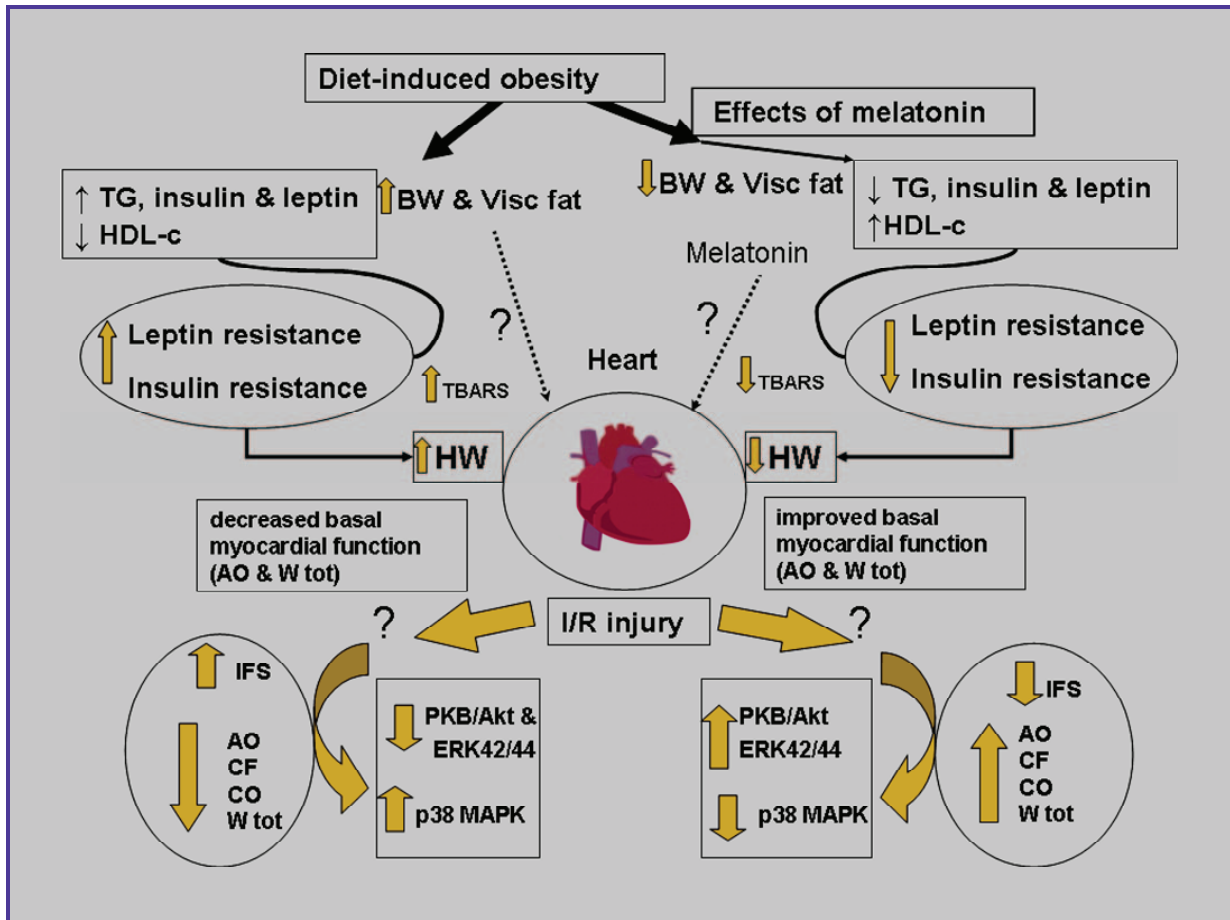


Figure 6- 1 Hypothetical representation of the effects of melatonin on the heart in diet-induced obesity.

Chronic melatonin consumption prevented the metabolic alterations associated with increased fat accumulation, insulin resistance and leptin resistance. It improved baseline and post ischaemic myocardial function and protected the heart against ischaemia and reperfusion injury. This was associated with an increased activation of PKB/Akt-ERK42/44 and a decreased activation of the pro-apoptotic kinase p38 MAPK during reperfusion. HW: heart weight; BW: body weight; Visc fat: visceral fat; I/R: ischaemia and reperfusion injury; AO: aortic output, CF: coronary flow; CO: cardiac output; IFS: infarct size; Wtot: work total; TG: triglycerides; HDL-c: high density lipoprotein-cholesterol. TBARS: thiobarbituric acid reactive substances.

6.2. FUTURE PERSPECTIVES

The present study demonstrated the beneficial effects of chronic melatonin treatment on the heart in obesity using the isolated heart, an *ex vivo* technique, without other physiological interference from other organs or functions. Thus, it is of paramount importance to test the present findings in an *in vivo* model. In addition, because of the time constraints we did not determine myocardial lipid peroxidation levels and apoptosis as well as functional recovery (following global ischaemia). The interactions between melatonin, leptin and insulin in obesity should receive further attention with particular attention to the signalling pathways involved. It is evident that the effects of melatonin should be evaluated in comprehensive clinical trials. The results obtained in this study suggest that melatonin should not only be taken prophylactically, but also by patients with ischaemic heart disease and in obesity. Melatonin is a cheap drug and can be purchased without prescription in South Africa. It therefore has enormous therapeutic potential in our society with its high prevalence of obesity and cardiovascular disease.

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